



Hemlock woolly adelgid alters fine root bacterial abundance and mycorrhizal associations in eastern hemlock



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ABSTRACT

While the impact of aboveground herbivores on plant biomass and fitness has received considerable attention, there has been far less research on the corresponding belowground impacts. The belowground effects of aboveground feeding may be particularly noticeable for invasive and/or outbreaking herbivore species that reach high densities and can cause major damage and sometimes death. The hemlock woolly adelgid, *Adelges tsugae*, is an invasive pest on the eastern seaboard of the United States that feeds on a native shade-tolerant conifer, the eastern hemlock *Tsuga canadensis*. Trees rapidly decline and die following infestation, and the invasion of this insect has devastated hemlock populations from Georgia in the south to Maine in the north. Despite their substantial impact on tree health, we are unaware of any research into the adelgid's effect on hemlock roots and the surrounding rhizosphere. We report the results of research assessing ectomycorrhizal root colonization, rhizosphere bacterial abundance, and root C:N ratios of infested and uninfested *T. canadensis*. We found that adelgid infestation decreased the percentage of root material colonized by ectomycorrhizal fungi by more than 67%. Rhizosphere bacterial abundance on fine roots was 25% lower on adelgid-infested versus uninfested trees, and roots of adelgid-infested trees contained significantly less carbon. Our results demonstrate that aboveground adelgid infestation can affect hemlock root composition and alter belowground interactions with ectomycorrhizal fungi and bacteria. This information demonstrates that above-belowground linkages can transmit the impact of herbivory far from the site of localized damage.

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1. Introduction

The impact of insect herbivores on plant growth and community structure can range from inconsequential to major; in extreme cases the structure and functions of entire ecosystems can be substantially altered (Lovett et al., 2006). In some instances, insect herbivores can increase biodiversity via preferential feeding on dominant species, allowing resources to be exploited by a greater number of species (Carson and Root, 2000). In other cases where herbivores inflict substantial damage, outbreaks of such species can devastate their hosts and cause major changes to the environment (Smith and Schowalter, 2001; Gandhi and Herms, 2010) and economic loss (Aukema et al., 2011; Oliveira et al., 2013).

Researchers are increasingly aware that herbivore grazing on aboveground green biomass can have profound belowground impacts on the composition of organisms and subsequent nutrient cycling in the rhizosphere (Bardgett and Wardle, 2010). The herbivore removal of aboveground plant tissue can alter patterns of carbon and nutrient allocation in belowground roots (Rasmann et al., 2009). This can affect the composition and abundance of rhizosphere-dwelling organisms. Cattle grazing, for instance, has been shown to alter microbial community composition and food web structure in the root zone of grass (Hamilton and Frank, 2001; Veen et al., 2010). Moose and snowshoe hare grazing have also led to reduced ectomycorrhizal (EM) colonization in roots of balsam poplar (*Populus balsamifera*) and willow (*Salix* spp.) (Rossow et al., 1997). These changes can in turn alter rates of nutrient cycling and availability in ways that affect the grazed plant. This feedback loop has been well-documented and can be surprisingly

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favorable for plant growth and recovery (Ruess and McNaughton, 1987; Krumins, 2014).

While the effect of folivory on aboveground–belowground interactions has been well-studied in herbaceous plants, the impact of sap-feeding herbivores on aboveground–belowground interactions in woody plant species has attracted less attention. This gap is notable in light of work documenting that sap-feeder impacts on woody plant fitness equal or exceed those of folivores (Zvereva et al., 2010). Aphid infestations, for instance, indirectly reduce root growth in Sitka spruce by limiting the tree's ability to provide carbon from photosynthesis (Day and Cameron, 1997) and reduce root tissue density of Douglas fir by inducing the translocation of additional carbohydrates from roots to shoots (Smith and Schowalter, 2001). Herbivory by the needle-feeding scale *Matsucoccus acalyptus* reduced EM colonization of pinyon pine (Gehring and Whitham, 1991); similarly, western spruce budworm (*Choristoneura occidentalis*) defoliation reduced EM colonization in Douglas fir seedlings (Kolb et al., 1999). More generally, both folivores and sap feeders can also impact epiphytic microbial communities through their production of nutrient-rich excrement (Stadler et al., 2001).

The hemlock woolly adelgid (*Adelges tsugae*; 'adelgid') is an invasive sessile herbivore that feeds exclusively on eastern hemlock (*Tsuga canadensis* (L.) Carr.) in the northeastern United States. While it has minimal impact on hemlock health in its native range of Japan and China (Havill et al., 2006), it can kill even mature eastern hemlocks in as little as four years; few heavily-infested trees survive longer than ten years (Orwig and Foster, 1998). While the impact of the adelgid on hemlock physiology has been studied (Radville et al., 2011; Gómez et al., 2012; Gonda-King et al., 2012; Domec et al., 2013), its effects on hemlock root physiology and the associated rhizosphere remain unexplored.

We report the results of work exploring the belowground impact of adelgid infestation on *T. canadensis*. We measured root EM associations of infested and uninfested hemlock trees, rhizosphere bacterial abundance, and root C:N ratios. Our findings demonstrate that aboveground adelgid infestation of eastern hemlock has belowground consequences that likely augment the ecosystem-level impact of this pest and may need to be addressed for the maximal success of forest restoration efforts.

2. Methods

As part of a long-term research program addressing the impacts of the hemlock woolly adelgid on eastern hemlock, we characterized adelgid infestation level and stand vigor in 79 stands in CT and 63 stands in MA. Following their initial characterization (1997–1998 in CT, 2002–2004 in MA; Orwig et al., 2002, 2012), these stands were repeatedly surveyed for adelgid infestation and stand vigor in 2005, 2007, 2009, and 2011 (Preisser et al., 2008, 2011). We assessed the rhizosphere of eastern hemlocks in a subset of the stands described above. In order to explore how adelgid infestation affects rhizosphere processes in eastern hemlock, we characterized both fine-root EM colonization (Study #1) and bacterial abundance (Study #2).

In Study #1, we quantified EM colonization of hemlock roots in three adelgid-infested and two uninfested hemlock stands in 2003; we focused our research on ectomycorrhizae as opposed to arbuscular (endo) mycorrhizae because conifers like hemlock are almost exclusively colonized by ectomycorrhizae (Smith et al., 1997). Infested stands were located in south-central Connecticut, and had been colonized by adelgid for 3–10 years; uninfested forests were located in central Massachusetts (Fig. 1). In Study #2, we quantified rhizosphere bacterial abundance from ten infested and ten uninfested hemlock stands in central and northern

Massachusetts in 2013 (Fig. 1). In both studies, trees sampled in the 'infested' treatment were heavily infested themselves (>1 mature adelgid/cm foliage growth) and surrounded by other heavily-infested trees. In contrast, trees sampled in the 'uninfested' treatments were in stands where no adelgid had been detected during previous large-scale surveys; to ensure that the sampled trees had maintained their uninfested status, we carefully surveyed each sampled tree and all trees within 10 m of it to ensure the absence of adelgid. Because of logistical constraints, we were unable to sample the same sites in both surveys. Regardless of location, all sampled stands consisted of >50% hemlock canopy cover within 100 m of the sampled trees, and 100% canopy cover within 10 m of the sampled trees.

2.1. Study 1: Ectomycorrhizal colonization of hemlock roots

From each of the five sites, we collected roots from 3 to 8 hemlock saplings (1.5–2 m in height); we sampled a total of 14 saplings from infested sites and 16 from uninfested sites. Soil and stand traits were sampled in the course of several related studies (Cobb et al., 2006; Orwig et al., 2013); infested sites averaged 708 ± 19 [SE] trees ha^{-1} , with a mean hemlock stand basal area of 46.6 ± 2.7 $\text{m}^2 \text{ha}^{-1}$; uninfested sites averaged 1072 ± 97 [SE] trees ha^{-1} , with a mean hemlock stand basal area of 45.6 ± 2.0 $\text{m}^2 \text{ha}^{-1}$. Soils in infested sites had a mean organic (forest floor) soil C:N ratio of $26.1 \pm 2.3\%$ [SE] and a mean mineral soil C:N ratio of $23.2 \pm 0.65\%$. Soils in uninfested sites had a organic (forest floor) soil C:N ratio of 26.9% and a mineral soil C:N ratio of 24.1% (also see online appendix #1). Only trees growing under a hemlock-dominated canopy were sampled. Each sapling was uprooted to expose the entire root system, and three 20 cm root samples per tree were collected and rinsed with deionized water (four of 30 sampled trees only had sufficient roots for two 20 cm root samples). Although we would have liked to sample mature trees, the labor involved in uprooting multiple large hemlock trees (necessary to ensure that the sampled roots in fact belonged to the chosen tree) necessitated using saplings. Each root sample was assigned a random number to ensure an unbiased assessment and then trimmed down to a 5 cm section. The grid intercept method (Giovannetti and Mosse, 1980) was used to assess the percent EM colonization for each root sample. Roots with EM colonization were differentiated on the basis of morphology, color, characteristics of the surface of the hyphal mantle, and planar views of different mantle layers using standard methods (Agerer, 1992). Each root sample was randomly dispersed in a 9 cm diameter petri plate with 0.5 cm grid lines. The intersection between grid lines and roots were designated as either EM-colonized or non-mycorrhizal. The proportion of root counts that were mycorrhizal was calculated for each root sample and averaged for each sapling. We took a total of 5902 counts, an average of 197 counts per tree (each tree had a minimum of 100 counts).

2.2. Study 2: Bacterial abundance in the rhizosphere

We collected fine roots (≤ 2 mm diameter; Robertson et al., 1999) from three understory hemlocks (2–5 m tall) at each of twenty sites (10 infested and 10 uninfested); all sampled trees were growing under a hemlock-dominated canopy. Again, our choice of the sampled trees was motivated by the difficulties inherent in uprooting multiple large hemlock trees (necessary to ensure that the sampled roots in fact belonged to the chosen tree). Infested sites averaged 1312 ± 240 [SE] trees ha^{-1} , with a mean hemlock basal area of 33.2 ± 1.5 $\text{m}^2 \text{ha}^{-1}$; uninfested sites averaged 859 ± 78 [SE] trees ha^{-1} , with a mean hemlock basal area of 38.2 ± 1.7 $\text{m}^2 \text{ha}^{-1}$. Soils in infested sites had a mean organic (forest floor) soil C:N ratio of $26.9 \pm 1.4\%$ [SE] and a mean mineral soil

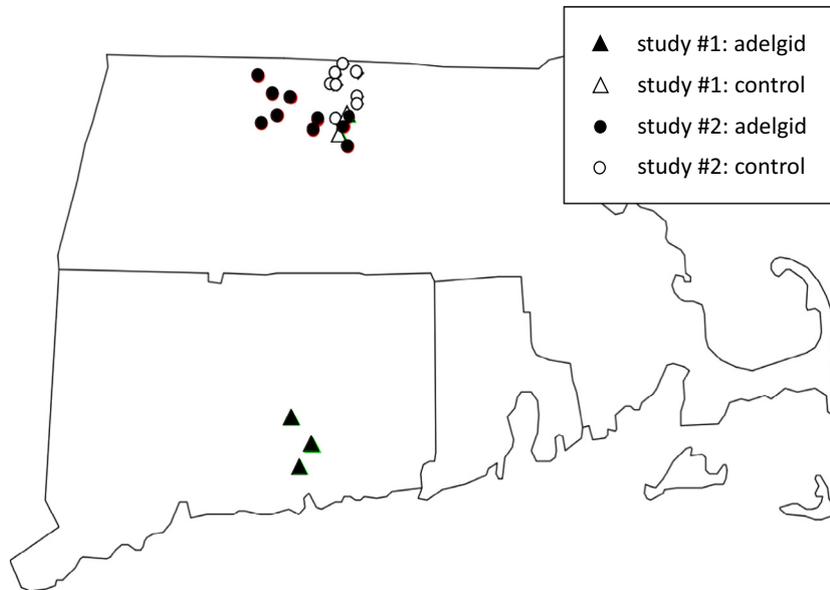


Fig. 1. Sites in Connecticut and Massachusetts used for the mycorrhizal (Study #1) and root bacterial abundance (Study #2) surveys.

C:N ratio of $24.5 \pm 2.0\%$. Soils in uninfested sites had a mean organic soil C:N ratio of $28.8 \pm 1.6\%$ and a mean mineral soil C:N ratio of $24.5 \pm 1.6\%$ (also see [online appendix #1](#); for a more detailed site description, see [Orwig et al., 2012](#)). Roots were collected from each hemlock by lightly scraping away leaf litter and organic soil from the base of the tree, extracting the roots, and clipping three 12–15 cm root samples. Root samples were combined in a single plastic bag (one bag per tree), immediately returned to the lab, and refrigerated for <2 h prior to fixation. Before fixing each sample, loose debris and soil was manually shaken off; ~ 0.1 g of fine roots from each tree was then placed in 5 ml of phosphate buffer saline (PBS) solution and vortexed for 2 min to suspend the bacteria. Each suspension was fixed with 1.5% filtered formalin (1.5% final formalin concentration) and again vortexed. Fixed samples were stored at 4 °C prior to staining and enumeration.

Staining took place within three days of the sample being fixed and collected; because the samples were fixed immediately after collection, there were no time-related differences between bacterial counts taken on different days. Bacterial abundance was determined using acridine orange direct counts (AODC) ([Kepner and Pratt, 1994](#)). Cell concentration was optimized by dilution to achieve countable samples. Between 0.1 and 0.5 ml of each fixed sample was removed (the extracted amount was supplemented with PBS to ensure a total volume of 1 ml) and then stained with 200 μ l of 0.1% acridine orange. Each sample was vacuum-captured onto a 0.2 μ m black polycarbonate filter (EMD Millipore Corporation, MA) and fixed to a slide with immersion oil. Enumeration was done using a Nikon Eclipse Ti inverted fluorescent microscope fixed with a Nikon DS-Fi1 camera. We determined mean bacterial abundance per slide by photographing ten randomly-chosen locations on each slide, using ImageJ to count bacterial densities, and averaging the ten counts. Each count thus reflects the mean number of cells per g fresh weight of plant material. Because the extraction procedures involved washing and removing fresh plant tissue, we could not calculate the dry weight of plant material.

We used the procedures outlined above to collect an additional fine root sample in 2013 for carbon and nitrogen analysis from each tree used for bacterial abundance at the 20 sites. Fine roots were rinsed with deionized water to remove soil particles and

dried at 60 °C in an oven for 48 h. Dried samples were ground into a fine powder using a grinding mill (Spex Mixer Mill 8000M, Metuchen, NJ) and analyzed for carbon and nitrogen content with a nutrient analyzer (Elementar vario MICRO cube, Mount Laurel, NJ). Two replicates of each fine root sample were analyzed, and the results averaged to calculate a site-level mean.

2.3. Statistical analysis

To test the effect of adelgid infestation on EM colonization, bacterial abundance, and root C:N, all samples were grouped by site and the infested and control sites were compared. Because the data from Study #1 did not meet the assumptions of normality, we analyzed it using a non-parametric median test; data from Study #2 was analyzed using one-way ANOVA. EM colonization was calculated as percent colonization and bacterial abundance was calculated as number of cells per gram fresh weight of root. All analyses were performed using JMP 9.0 (SAS, Cary, NC).

3. Results

The percentage of EM colonization was significantly lower ($10.6 \pm 2.4\%$ [SE]) on infested hemlock roots compared to roots from uninfested trees ($32.4 \pm 10.2\%$) (Median Test; $p = 0.046$). Hemlock fine root bacterial abundance, measured in millions of cells per gram, was also lower on infested versus uninfested trees ($F_{1,18} = 2.22$, $p = 0.044$; [Fig. 2A](#)). Chemical analysis of fine roots from adelgid-infested versus uninfested trees revealed that percent carbon was significantly lower in infested hemlock stands ($F_{1,18} = 5.11$, $p = 0.036$; [Fig. 2B](#)), but that adelgid infestation did not affect percent nitrogen ([Fig. 2C](#)). Despite the differences in root C, roots from infested versus uninfested stands did not differ in their root C:N ratio ([Fig. 2D](#)).

4. Discussion

We found that aboveground infestation by hemlock woolly adelgid significantly affected rhizosphere processes. The rhizosphere surrounding fine roots of adelgid-infested trees had less ectomycorrhizal colonization and lower bacterial abundance ([Fig. 2A](#)), while the fine roots themselves had lower carbon

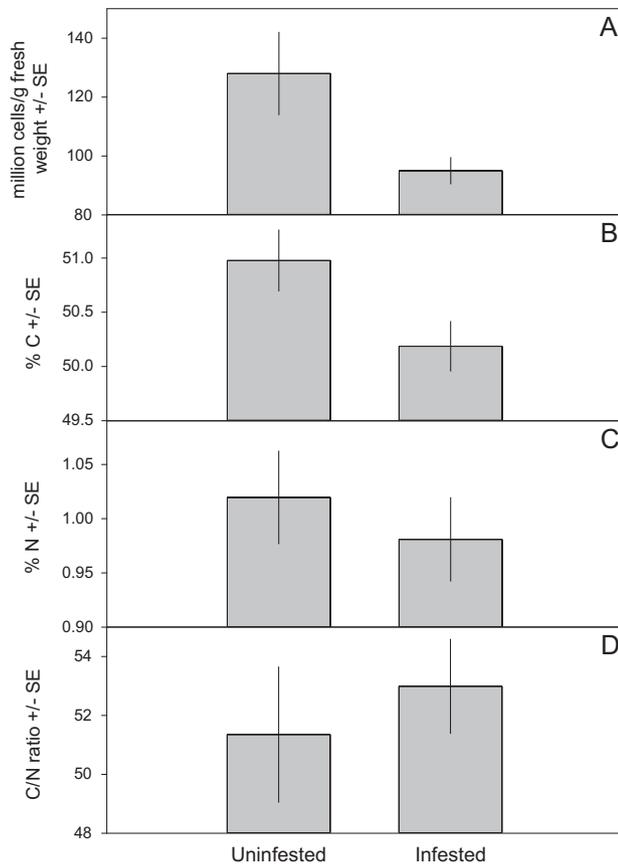


Fig. 2. Mean bacterial abundance (A), mean percent carbon (B), mean percent nitrogen (C), and mean carbon:nitrogen ratio (D) of eastern hemlock fine roots in 10 uninfested hemlock stands and 10 adelgid-infested hemlock stands.

concentrations (Fig. 2B). The lower percentage of ectomycorrhizal colonization found in our results is consistent with findings following herbivory from several types of insects (Gehring and Whitham, 1994b). One of the most common causes of decreased ectomycorrhizal colonization is herbivory-driven reductions in photosynthate availability; this can disrupt carbohydrate supply to mycorrhizae and reduce mycorrhizal root tip abundance (Gehring and Whitham, 1994b) and mycorrhizal inoculum potential (Lewis et al., 2008). Aphid outbreaks on other conifer species, for instance, can decrease photosynthetic efficiency (Day and Cameron, 1997). The hemlock woolly adelgid has been shown to cause similar reductions in photosynthesis (Nelson et al., 2014), and continued feeding on hemlock likely leads to continuous carbohydrate depletion. It is possible that the reduction in carbon found in the fine roots of infested trees (Fig. 2B) and lower EM colonization result from a disruption of that carbon source.

Environmental conditions can also play a significant role in the mycorrhizal response to herbivory (Bardgett and Wardle, 2003; Gehring and Whitham, 2003). There has been a documented decrease in EM colonization in sites with higher soil nutrient status compared with water- and nutrient-stressed sites (Gehring and Whitham, 1994a). Several environmental characteristics of chronic HWA infestation may have impacted the EM colonization response seen in this study. First, Stadler et al. (2005) and Stadler et al. (2006) provided evidence that adelgid impacts the composition of throughfall in infested stands. Their work showed higher inputs of N into the soil under adelgid-infested trees. Second, in addition to inputs from throughfall, adelgid damage often yields microenvironmental conditions that lead to increased soil N due to changes

in decomposition, N cycling and availability, and reduced tree uptake of nutrients (Kizlinski et al., 2002; Orwig et al., 2008). Enhanced soil N status resulting from either of these mechanisms may also have led to reduced colonization of hemlock fine roots and associated bacterial levels. Finally, altered mycorrhizal community structure resulting from increased N in the system may have selected for ectomycorrhizal species that perform less of a service for their host but still require the same carbon cost (Johnson, 1993).

We expected the abundance of bacterial cells colonizing the roots of adelgid-infested trees to be higher. Experiments in grasslands ecosystems showed that grazed plants exude more carbon into the rhizosphere, thus stimulating microbial growth and metabolism (Hamilton and Frank, 2001). The priming effect (Kuzyakov et al., 2000) stimulated by above-ground herbivory fed back positively to the grasses, increasing nutrient availability. Rhizosphere microbes have the primary responsibility for making nutrients available to plants through the decomposition and mineralization of soil organic matter (Vessey, 2003). It is interesting to note that we did not find increased microbial abundance in the trees we studied. This difference may be due to important differences in the feeding behavior of herbivores and how they affect the host plant (Lovett et al., 2006). Sucking insects, for instance, export far less wasted plant biomass (increasing soil organic matter) and frass to the forest floor than do chewing insects or larger grazers (Zvereva et al., 2010).

Bacterial and fungal communities are often tightly coupled in the rhizosphere. The reduced bacterial abundance found in adelgid-infested hemlock stands may simultaneously be linked to reduced ectomycorrhizal associations and changes in root and soil nutrient chemistry associated with infestation. Indeed, some studies have found that soil communities experiencing mycorrhizal loss also lose their fungally-associated bacteria (Hol et al., 2014). However, the significant decline in absolute numbers of bacteria suggests resource limitation from the root. The implications of decreased bacterial load will be decreased mineral nutrient availability to the tree (Vessey, 2003; Wardle et al., 2004); this is consistent with our results (Fig. 2A). Knowing both the load of EM and bacteria in the rhizosphere is, however, only the first critical step. Soil communities are classically divided into bacterial- and fungal-based energy channels (Moore and Hunt, 1988; De Ruiter et al., 1995). It is the balance of these two energetic pathways, however, that leads to stability and functioning of the rhizosphere community (Rooney et al., 2006) and overall plant health. Therefore, future work will test differences in community composition of both mycorrhiza and bacteria colonizing the roots of trees in affected and unaffected sites. The reduced colonization by EM and bacterial abundance found here may be caused by strong competitors dominating the rhizosphere community.

Our work provides the first documentation of the below-ground consequences of above-ground herbivory on eastern hemlock by an exotic herbivore. The impact of aboveground feeding by the hemlock woolly adelgid on the rhizosphere processes of the hemlock and the mycorrhizal and bacterial abundances illustrates the need for a greater understanding of how herbivores impact all aspects of an ecosystem. From a management perspective, there is increased recognition (Kardol and Wardle, 2010) of the importance of aboveground–belowground linkages in determining the efficacy of management and restoration efforts. Specifically, adelgid-mediated alterations in the belowground communities that facilitate hemlock growth could make it more difficult to replant hemlocks in formerly-suitable areas. Further research should investigate the soil community structure of infested hemlock stands against uninfested hemlock stands, to see if there are shifts in the species found in addition to the change in abundance of bacteria.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foreco.2014.12.010>.

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