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Chad M. Rigsby
Ian G. Kinahan
Amelia May
Amy Kostka
Nick Houseman

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Authors
Chad M. Rigsby, Ian G. Kinahan, Amelia May, Amy Kostka, Nick Houseman, Suzanne K. Savage, Elizabeth R. Whitney, and Evan L. Preisser
Impact of hemlock woolly adelgid (*Adelges tsugae*) (Hemiptera: Adelgidae) infestation on the jasmonic acid-elicited defenses of eastern hemlock (*Tsuga canadensis*)

Chad M. Rigsby¹,²,³, Ian G. Kinahan¹, Amelia May¹, Amy Kostka¹, Nick Houseman¹, Suzanne K. Savage¹, Elizabeth R. Whitney¹, and Evan L. Preisser¹

¹Department of Biological Sciences, The University of Rhode Island, Kingston, RI, USA
²Author for correspondence: crigsby@bartlettlab.com
³Current Address: Bartlett Tree Research Laboratories, The Morton Arboretum, Lisle, IL, USA
Abstract

Hemlock woolly adelgid (‘HWA’) is an invasive piercing-sucking insect in eastern North America, which upon infestation of its main host, eastern hemlock (‘hemlock’), improves attraction and performance of folivorous insects on hemlock. This increased performance may be mediated by HWA feeding causing antagonism between the the jasmonic acid and other hormone pathways. In a common garden experiments using HWA infestation and induction with methyl jasmonate (MeJA) and measures of secondary metabolite contents and defense-associated enzyme activities, we explored the impact of HWA feeding on the local and systemic induction of JA-elicited defenses. We found that in local tissue HWA or MeJA exposure resulted in unique induced phenotypes, while the combined treatment resulted in an induced phenotype that was a mixture of the two individual treatments. We also found that if the plant was infested with HWA, the systemic response of the plant was dominated by HWA, regardless of whether MeJA was applied or not. Interestingly, in the absence of HWA, hemlock plants had a very weak systemic response to MeJA. We conclude that HWA infestation prevents systemic induction of JA-elicited defenses. Taken together, compromised local JA-elicited defenses combined with weak systemic induction could be major contributors to increased folivore performance on HWA-infested hemlock.

Key Words: induced defenses, systemic induction, invasive forest pest, herbivory, conifers

Introduction
Plants growing under the resource-limited conditions typical of natural systems must choose how to allocate scarce resources to functions such as growth, reproduction, and defense. The induction of chemical and physical defenses in response to herbivore or pathogen attack is hypothesized to be an energetically advantageous solution to such dilemmas (Baldwin 1998; Gómez et al. 2007). When attacked by mobile herbivores that can respond to local defense by seeking out undefended plant biomass, plants can respond via systemic responses that stimulate defense induction in both damaged and undamaged tissues (Kant et al. 2015). Because they incur energetic costs in tissue that has not yet been – and might not be – attacked, systemic defenses are often considered a bet-hedging strategy: the cost of systemic induction is roughly half the cost borne by non-systemically-induced plants that are attacked (Reynolds et al. 2019).

Systemic induction can be influenced by vascular architecture and connectivity, plant size and age, and volatile production (Orians 2005; Kant et al. 2015). Several endogenous compounds that act as systemic signals include phytohormones, peptides, and volatile compounds (Kant et al. 2015). Jasmonates appear particularly important for systemic defense induction (Heil and Ton 2008). Precursors to jasmonic acid (JA) conjugates, such as JA itself, are produced at the site of herbivore attack and transported through the phloem to undamaged tissues (Fürstenberg-Hägg et al. 2013). JA-elicited systemic defense expression requires both JA biosynthesis at the site of damage and JA perception in distant undamaged tissues (Heil and Walters 2009). A substantial set of literature has demonstrated the antagonistic relationship between salicylic acid (SA) and JA where the SA-induced monomerization of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) suppresses JA biosynthesis and inhibits JA-responsive genes (Beckers and Spoel 2006). This antagonistic relationship suggests that the expression of JA-
elicited systemic defense in distal plant tissues would be compromised if locally-produced SA interfered with JA biosynthesis at the attack site.

Hemlock woolly adelgid (HWA; *Adelges tsugae* Annand) is a sessile, stylet-feeding insect that is invasive to eastern North America. It has caused mass mortality of eastern hemlock (‘hemlock’; *T. canadensis* L.) within its invaded range. Chronic HWA infestation causes a ‘hypersensitive-like’ response in hemlock that is characterized by the accumulation of SA, hydrogen peroxide (H$_2$O$_2$), and proline and increases in methyl salicylate (MeSA) emissions (Radville et al. 2011; Gómez et al. 2012; Pezet et al. 2013; Pezet and Elkinton 2014 Schaeffer et al. 2018; Rigsby et al. 2019). The nature of this response led to the hypothesis that HWA infestation would increase host quality for JA-eliciting herbivores by decreasing the induction of JA-linked plant defenses. Consistent with this scenario, Wilson et al. (2016) reported increased performance of hemlock looper (*Lambdina fiscellaria*) on HWA-infested hemlock, and Kinahan et al. (2020) found increased gypsy moth (*Lymantria dispar*) larval preference for and performance on HWA-infested hemlocks in both field and laboratory settings.

Although the latter two studies are consistent with the hypothesis that HWA-mediated increases in SA disrupt JA-based plant defense, this linkage has not been experimentally confirmed. While changes in the inducibility of JA-elicited defenses may be involved, SA- and JA-elicited defense responses are remarkably similar in hemlock (Rigsby et al. 2019). In an experiment that used HWA and gypsy moth larvae to directly induce SA- and JA-elicited responses, Rigsby et al. (*in review*) found that both HWA and gypsy moth increased foliar SA levels; simultaneous herbivory by both insects had an additive effect. Gypsy moth herbivory resulted in accumulation of JA and JA-Ile, the active form of JA, while HWA inhibited the ability of gypsy moths to elicit JA accumulation (Rigsby et al. *in review*). These findings support
the hypothesis that HWA infestation prevents hemlock from accumulating JA phytohormones in response to JA-eliciting herbivores. Intriguingly, however, HWA infestation also increased accumulation of several bioactive gibberellins (GAs), hormones known to play a critical role in plant growth (i.e., stem elongation and leaf expansion; Davière and Achard 2013). This HWA-elicited GA accumulation is notable because GAs are also known to antagonize JA signaling (de Lucas et al. 2008). This result suggests that JA accumulation and the elicitation of JA-linked defenses could be compromised by one or both of these mechanisms.

Previous research addressing herbivore-herbivore interactions in the HWA/hemlock system has focused on local plant defense induction (i.e., changes occurring at the site of plant damage); the impacts of HWA on systemic defense induction have not been addressed. We present the results of work assessing the potential for HWA-induced suppression of JA-elicited systemic defense induction. Using a common garden planting that contained both HWA-infested and HWA-free hemlock saplings, we induced stems with methyl jasmonate (MeJA), a methylated form of JA whose topical application induces JA-elicited responses in hemlock (Rigsby et al. 2019). We evaluated induction responses by quantifying chemical and physiological defensive responses (e.g., total soluble phenolics, peroxidase activity, etc) in foliage on stems directly sprayed with MeJA and needles not directly sprayed, but on the same branch. We hypothesized that (1) HWA infestation would attenuate local MeJA-elicited defense responses, in accordance with Rigsby et al. (2019), but would completely shut down MeJA-elicited systemic responses. Conversely, we predicted that (2) both local and systemic responses would be uninhibited in HWA-free plants.

Materials and Methods
**Hemlock common garden, treatments, and sampling.** In early spring 2014, 350 herbivore-free hemlock saplings (0.5-0.7 m tall) that were grown from seed collected in Pennsylvania and had not been treated with insecticides were purchased from Vans Pines Nursery (West Olive, MI). The 320 healthiest of these trees were planted in five 64-tree blocks (eight rows and columns with trees spaced 1-1.5 m apart) into the understory of a mixed hardwood stand at the Kingston Wildlife Research Station (South Kingstown, RI) in April 2014. As part of ongoing experiments in our laboratory, a subset of trees within each block were randomly selected for artificial infestation with HWA, performed every year at approximately mid-spring (timed with crawler emergence). Briefly, we cut HWA-infested stems from naturally growing hemlocks located less than one km from our experimental site, inspected this foliage for the presence of only HWA, and secured this cut foliage to each hemlock within this treatment using wire to secure this cut foliage to each hemlock (see Butin et al. 2007 for detailed methods). Trees in the control treatment were sham-inoculated with herbivore-free foliage to control for inoculation-related disturbance. The uninfested status of each control tree was confirmed via careful visual inspection of each tree prior to the removal of any foliage. Trees were protected from herbivory and treatment cross-contamination with chicken-wire cages covered in mesh bags (Agribon-15, Johnny’s Selected Seeds, Waterville, ME, USA; 90% light transmission).

Twelve trees from each of the two treatments (HWA-infested, uninfested controls) were selected so that each treatment was represented by at least three trees in each of four spatial blocks; trees from the fifth spatial block was excluded because this block was much shadier than the other four. A single branch was selected on each tree; all sampled branches were of similar length and diameter (ANOVA; $P > 0.05$ for all) and the branches from HWA-infested trees had moderate, but roughly equivalent HWA densities (0.5-1 HWA cm$^{-1}$ stem). Each branch was
marked by placing flagging placed at its base (Fig. 1). Twice weekly for a two-week period (28 Aug - 7 Sept 2017), an elicitor solution containing 10 mM MeJA in a carrier solution of 0.1% (v:v) Tween 20 (MeJA treatment) or carrier solution only (control treatment) was carefully applied using a fine-tipped paint brush, so that MeJA solution did not run off, to the first lateral stem proximal to the terminal stem, near the flagging. All treated branches were harvested on 11 Sept, placed in aluminum foil, and stored at -80°C. In order to understand how HWA impacts systemic defense signaling, we harvested a stem immediately proximal (denoted as “Systemic” stem) to the treated stem (denoted as “Local” stem) (Fig. 1). This resulted in four treatment combinations (HWA +/- and MeJA +/-; n = 6 biological replicates per treatment combination; 24 total), with two location categories per branch: “Local” and “Systemic” stems (48 total samples; Fig. 1). Lastly, in order to eliminate additional sources of variation, only foliage produced in the current growing season (i.e., newly produced foliage) was used in this study, foliage that was produced prior to the season of our experiment was not used in this study (Fig. 1).

*Chemical and Physiological Analyses.* Crude levels of chemical defenses were quantified as described in Rigsby et al. (2019); any deviations from these protocols are detailed below. Briefly, needles were ground into a powder in liquid nitrogen using a mortar and pestle and 100 mg were placed in a 2 ml microtube. Tissue was twice-extracted in 0.5 ml HPLC-grade methanol. Following centrifugation at 16,000 g (10 min, 4°C), the supernatants were combined. Methanol-soluble terpene content was quantified immediately using chloroform and H₂SO₄ (Rigsby et al. 2019) with linalool as the standard. Soluble phenolic content was quantified via the Folin-Ciocalteu method using chlorogenic acid as standard; proanthocyanidin content was quantified using the acidified butanol method (Rigsby et al. 2019). Chlorogenic acid was used as a standard for the quantification of soluble phenolics because prior research found that
chlorogenic acid dominates the soluble phenolic profile of hemlock foliage (Rigsby et al. 2020). The cell wall-bound phenolic (CW-bound phenolic) and lignin contents were determined as per Rigsby et al. (2019) using gallic acid and spruce lignin, respectively, as the standard. Hydrogen peroxide ($\text{H}_2\text{O}_2$) was quantified according to the KI method (Junglee et al. 2014; Rigsby et al. 2019).

For enzyme activity assays, 200 mg needle powder was extracted on ice in five volumes of extraction buffer (50 mM NaPO$_4$, pH 6.8, 10% PVPP, 5% Amberlite XAD4 resin, and 1 mM EDTA) and the 10,000 g supernatant was used as the source of enzymes. Chitinase (CHI) and lipoxygenase (LOX) activities were quantified according to Rigsby et al. (2016) using chitin azure (OD$_{575}$ mg$^{-1}$) and linoleic acid ($\mu$moles min$^{-1}$ mg$^{-1}$), respectively, as substrates. Peroxidase (POX) activity was quantified according to Rigsby et al. (2018) using guaiacol and $\text{H}_2\text{O}_2$ as substrates ($\mu$moles min$^{-1}$ mg$^{-1}$). Phenylalanine ammonia lyase (PAL) activity was quantified by monitoring the conversion of L-phenylalanine to trans-cinnamic acid (Chen et al. 2006; nmoles hr$^{-1}$ mg$^{-1}$). To express enzyme activities per unit protein, the protein content of extracts was determined using the Bradford (1976) method with bovine serum albumin as standard. During preliminary experiments, we attempted to detect polyphenol oxidase activity using multiple substrates, as well as trypsin inhibitor activity, but were unable to do so.

**Statistical analyses.** The effect of HWA, MeJA, branch position (i.e., systemic induction), and their interactions on relative metabolite levels and enzyme activities was assessed using an ANOVA with stem position nested within tree identity. An ANCOVA was initially used with block as a covariate; because block was never significant, we proceeded with ANOVAs. We were interested in detecting (1) within-treatment differences in foliar position (i.e., ‘Local’ vs. ‘Systemic’ within a single treatment combination) and (2) between-treatment
differences for a given foliar position (i.e., ‘HWA-/MeJA-’ vs. ‘HWA+/MeJA-’ vs. ‘HWA-/MeJA+’ vs. ‘HWA+/MeJA+’ within a single sampling position). For post-hoc comparisons of within-treatment differences between sampling positions, we used $t$-tests to directly compare Local and Systemic foliage. For post-hoc comparisons of treatment combinations within a sampling position, we first performed $t$-tests comparing all combinations of interest, then the resulting $P$-values were adjusted via the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). For example, if comparing all four treatment combinations of ‘Local’ foliage, the six calculated $P$-values were included in the Benjamini-Hochberg correction. Because different sampling positions from different treatments were not of interest (e.g., ‘Local’ foliage from ‘HWA+/MeJA-’ vs. ‘Systemic’ foliage from ‘HWA-/MeJA-’), these comparisons were not made. These post-hoc procedures was only used if significant interactions between treatments/sampling locations were detected. All statistical analyses were performed in R (R Development Core Team 2020).

Results

Secondary Metabolites. For both Local and Systemic foliage, CW-bound phenolics, lignin, and H$_2$O$_2$ all had increased tissue levels as a result of HWA infestation relative to uninfested controls (Figs. 2A, 2B, and 2C, respectively). The application of MeJA had no effect on CW-bound phenolic or H$_2$O$_2$ contents in either Local or Systemic foliage (Figs. 2A and 2C, respectively), but did cause lignin to accumulate in Local foliage in the absence of HWA. However, this lignin accumulation was attenuated in the presence of HWA in Local foliage (Fig. 2B). Foliage position (i.e., “Local” vs. “Systemic” foliage) had a significant effect on lignin and H$_2$O$_2$ contents. In the absence of HWA, MeJA application (HWA-/MeJA+) significantly increased lignin content in Local foliage but not in adjacent Systemic foliage (Fig. 2B).
Additionally, in the presence of HWA and when MeJA was applied (HWA+/MeJA+), H₂O₂ content was significantly greater in Systemic than in Local foliage (Fig. 2C). There were no elicitor treatment or sampling location effects for soluble phenolics (71.70 ± 1.05 mg g⁻¹ DW), proanthocyanidins (33.54 ± 1.32 OD₅₅₀ g⁻¹ DW), or methanol-soluble terpenes (14.05 ± 0.15 mg g⁻¹ DW).

**Defensive Enzyme Activities.** In both Local and Systemic foliage, HWA infestation increased POX activity (Fig. 3A), while the application of MeJA increased LOX and PAL activities in Local foliage, only (Figs. 3B and 3C, respectively). Interestingly, infestation by HWA had no effect on the MeJA-elicited increase in LOX activity in Local foliage (i.e., the increase in LOX activity caused by MeJA application was not attenuated by the presence of HWA in Local foliage). However, this was the case for PAL activity, as HWA infestation severely inhibited the MeJA-elicited increase in PAL activity in Local foliage (Fig. 3C). Foliage position (i.e., “Local” vs. “Systemic” foliage) had a significant effect on both LOX and PAL activities. As with lignin content, the increase in LOX and PAL activities that were found in Local foliage in the absence of HWA and with MeJA application (HWA-/MeJA+), did not occur in Systemic foliage (Figs. 3B and 3C). This was also the case for LOX activity in the presence of HWA and with MeJA application (HWA+/MeJA+), where MeJA application resulted in increased activity in Local but not in Systemic foliage (Fig. 3A). There were no elicitor treatment or sampling location effects for CHI activity (0.31 ± 0.01 OD₅₇₅ mg⁻¹).

**Discussion**

The systemic induction of defenses is considered an important bet-hedging strategy for plants to minimize fitness costs (Reynolds et al. 2019), and systemic induction is viewed as an adaptive response against herbivores that impose chronic injury, continually increase populations
on individual plants, and/or can move among plant parts (Mason et al. 2017). Like many woody plants, an abundance of folivorous insects utilize hemlock as a host resource, including a variety of leafminers, loopers, leafrollers, budworms, needleworms, tussock moths, cutworms, and others (Maier et al. 2011). Recent research has shown that HWA infestation increases the attraction to and performance of folivorous insects on hemlock (Wilson et al. 2016; Rigsby et al. 2019; Kinahan et al. 2020), and this increase in folivore performance may be facilitated by the compromising of JA-elicited defenses locally at the site of folivore attack (Rigsby et al. 2019; 2020). This study sought to investigate the impact of HWA infestation on the induction of systemic, JA-elicited defenses. We hypothesized that (1) the HWA-instigated attenuation of local JA induction would be accompanied by a complete lack of systemic responses, and that (2) systemic responses would occur on HWA-free plants.

With regards to our first hypothesis that HWA infestation would attenuate local MeJA-elicited defense responses, our data somewhat agree with this though defenses presented rather as a blend between HWA-induced and MeJA-induced responses. This was consistent with previous research that found local JA-elicited defense expression is altered by HWA infestation (Rigsby et al. 2019). The second part of the hypothesis, that this local attenuation would be accompanied by complete inhibition of systemic responses, which also appears to be supported generally as systemic defense expression was completely masked by the local response to HWA infestation. Patterns of metabolite accumulation and enzyme activities of this treatment-position combination (i.e., systemic HWA+/MeJA+) was most similar to both the local and systemic HWA+/MeJA- treatments. Even if JA-elicited host responses were not locally compromised, the lack of systemic responses to mobile herbivores would pose a serious problem for a woody plant, as mobile folivores could simply move to these undefended tissues (Mason et al. 2017).
The apparent lack of systemic induction by MeJA+ plants was unanticipated and the opposite of our second hypothesis. Several variables could have contributed to this, such as interspecific species variation in systemic inducibility (e.g., Heil and Ploss 2006), site conditions (e.g., shade is known to inhibit JA responses; Cipollini 2004), MeJA dose (e.g., Naidoo et al. 2013), and/or vascular architecture (e.g., the stems chosen for our experiment may not have been as connected as we perceived; Orians 2005). However, the differential responses of LOX and PAL activities in the systemic tissues was particularly interesting (Figs. 3A and 3C). The activity of LOX, which should be an excellent of JA-elicitation indicator as it is directly involved in JA synthesis (Beckers and Spoel 2006) and directly (Felton et al. 1994) and indirectly (War et al. 2012) involved in defense, was not increased systemically by MeJA. However, PAL activity was increased systemically with MeJA application, perhaps demonstrating that PAL activity may better indicate JA-elicitation than LOX activity. Regardless of this, systemically increased PAL activity indicates that some sort of signal likely made it to this stem and was perceived by these tissues.

Interestingly, we did not detect local or systemic accumulation of soluble phenolics, including proanthocyanidins, and methanol-soluble terpenes. These classes of secondary metabolites are known to be critically important anti-herbivore defenses in conifers (Raffa et al. 2017). Previous research showed significant, positive effects of both HWA infestation and MeJA application on soluble phenolic content, including proanthocyanidins (Rigsby et al. 2019). Similar levels of CHI activity across all treatment combinations was also unanticipated, since previous research found that the activity of this enzyme was strongly enhanced by HWA infestation and MeJA application (Rigsby et al. 2019). In agreement with this previous research, we detected accumulation of CW-bound phenolics and H₂O₂, and increases in POX activity in
response to HWA infestation, and a strong positive effect of MeJA application on LOX activity (Rigsby et al. 2019). One difference between these two experiments is that Rigsby et al. (2019) used potted hemlocks in full sun while this study used hemlocks planted in the understory of a mixed hardwood stand. It may be that some aspect(s) of these environmental differences had some effect on hemlock response to our treatments. In addition to normal growth, GAs are also associated with shade-avoidance and growth, and JA pathways interact directly and antagonistically through DELLA-JAZ interactions (Wasternack and Hause 2013; Davière and Achard 2016), and shaded plants are often unable to activate JA-elicited responses (Cipollini 2004). HWA has a positive effect on a few major GAs (Rigsby et al. In Review), and the addition of shade may further increase gibberellin accumulation and antagonization of the JA pathway. An additive or synergistic effect between HWA infestation and shade on the inducibility of JA-elicited defenses would have major impacts on hemlock herbivore interactions, including between hemlock and HWA, itself. It has been noted by many researchers and practitioners that HWA appears to perform substantially better on its host when hemlock is shaded (Hickin and Preisser 2015).

The systemic induction of defenses is thought to be an important strategy of plants to reduce fitness costs (Kant et al. 2015; Reynolds et al. 2019), especially against herbivores that can move between plant tissues (Mason et al. 2017). Field observations and laboratory assays have shown dramatic increases in host quality and attraction to these kinds of herbivores (Wilson et al. 2016; Rigsby et al. 2019; Kinahan et al. 2020). In this study, we found that host responses to HWA infestation essentially overwhelm and prevent JA-elicited systemic defense expression, but we also detected very little JA-elicited systemic responses in hemlock in the absence of HWA. We conclude that in the absence of HWA, some JA-associated signal may be translocated
and systemically perceived, as evidenced by significantly elevated PAL activity. Environmental conditions of our experiment may have played a role in this lack of response, however, hemlock often exists in the environment in dense, shaded conditions (Hadley 2000), still allowing our results to be ecologically meaningful. Future research should explore the role of shade on local and systemic SA- and JA-elicited responses in hemlock.

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Fig. 1. Positioning of “Local” and “Systemic” stems used in Experiment 2. Local stems directly received either 10 mM MeJA in 0.1% (v:v) Tween-20 or control solution (0.1% Tween-20) and Systemic stems received no treatment.
Fig. 2. Mean (± SEM) cell wall-bound phenolics (A), lignin (B), and hydrogen peroxide (C) contents of Local (left set of four bars) and Systemic (right set of four bars) foliage infested with hemlock woolly adelgid (+ HWA, hatched right two bars) or not (- HWA, unhatched left two bars) and/or treated with methyl jasmonate (+ MeJA, black bars) or not (- MeJA, white bars). Significant treatment and interaction effects are listed for each response. Different uppercase letters indicate significant differences within foliage position and different lowercase Greek letters indicate significant differences between foliar positions within a treatment combination.

For cell wall-bound phenolics (A), MeJA \( (F_{1,36} = 1.0; \ P = 0.317) \), HWA x MeJA \( (F_{1,36} = 0.6; \ P = 0.453) \), stem position \( (F_{2,36} = 1.1; \ P = 0.336) \), HWA x stem position \( (F_{2,36} = 0.0; \ P = 0.971) \), MeJA x stem position \( (F_{2,36} = 0.2; \ P = 0.836) \), and HWA x MeJA x stem position \( (F_{2,36} = 0.3; \ P = 0.726) \) were all not significant predictors. For lignin (B), only MeJA x stem position \( (F_{2,36} = 0.8; \ P = 0.455) \) was not a significant predictor. For hydrogen peroxide (C), MeJA \( (F_{1,36} = 2.8; \ P = 0.103) \), HWA x MeJA \( (F_{1,36} = 1.4; \ P = 0.252) \), HWA x stem position \( (F_{2,36} = 1.7; \ P = 0.193) \), MeJA x stem position \( (F_{2,36} = 1.0; \ P = 0.372) \), and HWA x MeJA x stem position \( (F_{2,36} = 0.2; \ P = 0.805) \) were all not significant predictors.
Fig. 3. Mean (± SEM) lipoxygenase (A), peroxidase (B), and phenylalanine ammonia lyase (C) activities of Local (left set of four bars) and Systemic (right set of four bars) foliage infested with hemlock woolly adelgid (+ HWA, hatched right two bars) or not (- HWA, unhatched left two bars) and/or treated with methyl jasmonate (+ MeJA, black bars) or not (- MeJA, white bars). Significant treatment and interaction effects are listed for each response. Different uppercase letters indicate significant differences within foliage position and different lowercase Greek letters indicate significant differences between foliar positions within a treatment combination. For lipoxygenase activity (A), HWA ($F_{1,36} = 0.9; \ P = 0.357$), HWA x MeJA ($F_{1,36} = 0.4; \ P = 0.526$), HWA x stem position ($F_{2,36} = 1.5; \ P = 0.227$), and HWA x MeJA x stem position ($F_{2,36} = 0.1; \ P = 0.915$) were all not significant predictors. For peroxidase activity (B), MeJA ($F_{1,36} = 1.2; \ P = 0.282$), HWA x MeJA ($F_{1,36} = 0.4; \ P = 0.535$), stem position ($F_{2,36} = 0.2; \ P = 0.785$), HWA x stem position ($F_{2,36} = 1.1; \ P = 0.334$), MeJA x stem position ($F_{2,36} = 0.8; \ P = 0.473$), and HWA x MeJA x stem position ($F_{2,36} = 1.4; \ P = 0.253$) were all not significant predictors. For phenylalanine ammonia lyase activity (C), HWA ($F_{1,36} = 3.1; \ P = 0.088$), MeJA x stem position ($F_{2,36} = 0.0; \ P = 0.966$), and HWA x MeJA x stem position ($F_{2,36} = 1.9; \ P = 0.162$) were all not significant predictors.