Agricultural and Forest Entomology

Agricultural and Forest Entomology (2019), **21**, 227–234

DOI: 10.1111/afe.12324

Conifer responses to a stylet-feeding invasive herbivore and induction with methyl jasmonate: impact on the expression of induced defences and a native folivore

Chad M. Rigsby*[1], Emma E. Shoemaker*, Mary M. Mallinger*, Colin M. Orians† and Evan L. Preisser*

**Department of Biological Sciences, The University of Rhode Island, Kingston, RI 02881, U.S.A. and †Department of Biology, Tufts University, Medford, MA 02114, U.S.A.*

Abstract

1 Trees attacked by multiple herbivores need to defend themselves against dynamic biotic challenges; appropriate responses to one stressor can elicit hormonal responses that are antagonistic to another. Hemlock (*Tsuga canadensis*) infestation by hemlock woolly adelgid (HWA; *Adelges tsugae*) results in the accumulation of the defensive hormone salicylic acid.

2 We explored the potential for HWA infestation to interfere with anti-folivore-induced defence signalling and its implications for a native folivore (hemlock looper; *Lambdina fiscellaria*). Hemlocks were infested with HWA and/or sprayed with methyl jasmonate; foliar defences were analyzed and foliage quality for looper larvae was assessed.

3 Both treatments activated foliar defensive traits, including a HWA-mediated increase in peroxidase activity and an accumulation of cell wall-bound phenolics and lignin, as well as a methyl jasmonate-mediated increase in lipoxygenase activity. The two treatments had an additive effect on other defensive traits and both treatments negatively affected looper performance.

4 These results suggest that salicylic acid and jasmonic acid are not strictly antagonistic in conifers and that both have a role in anti-folivore defence signalling. The present study illustrates the need for a better understanding of hormone signalling, cross-talk and induced responses in conifers.

Keywords conifers, defence induction, induced defence signalling, SA–JA antagonism, stylet-feeders.

Introduction

Conifers (Pinaceae) often dominate temperate, alpine and boreal forests in the northern hemisphere (Ralph *et al.*, 2006). This family includes genera of major ecological and economic importance, such as pine (*Pinus*), spruce (*Picae*), hemlock (*Tsuga*) and fir (*Abies*), and the ecological success of many conifer species is considered to be linked to their effective defences against natural enemies (Bonello *et al.*, 2006; Krokene, 2015). The energetic costs of these anti-herbivore responses make it important that plants be induced only when appropriate (Baldwin, 1998). In conifers, for example, the accumulation of terpene and phenolic metabolites induced by bark beetle (Coleoptera: Curculionidae) attacks can substantially improve the likelihood of host survival (Schiebe *et al.*, 2012). Aside from a few specific systems (the pine processionary moth; *Thaumetopoea pityocampa*), most research addressing induced defence responses in conifers has focused on pine and spruce interactions with bark beetles; less attention has been paid to defence against other herbivorous insects (Ralph *et al.*, 2006; Eyles *et al.*, 2010).

When multiple herbivore species are present, the responses induced by one herbivore can affect co-occurring species. There are multiple examples of herbivores from different feeding guilds (e.g. leaf-chewing, stylet-feeding) indirectly affecting each other via their impact on plant physiology (Soler *et al.*, 2012). The phytohormones jasmonic acid (JA) and salicylic acid (SA) play a central role in these induced plant defences. Chewing insects such as caterpillars are generally assumed to trigger the

Correspondence: Chad M. Rigsby. e-mail: crigsby@bartlettlab.com

[1] Present address: Bartlett Tree Research Laboratories, Charlotte, NC 28278, U.S.A.

JA pathway, whereas stylet-feeding insects often elicit the SA pathway (Morkunas *et al.*, 2011). Research has demonstrated positive interactions (cross-talk) and antagonism between these induced-response pathways that prevent plants from responding simultaneously to SA- and JA-elicited challenges (Kroes *et al.*, 2015). However, such studies have mostly been conducted using herbaceous model plants such as *Arabidopsis*, tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*) (Preston *et al.*, 1999; O'Donnell *et al.*, 2003; Cipollini *et al.*, 2004; Thaler *et al.*, 2012).

Much less attention has been paid to woody plants. Although SA–JA antagonism has been demonstrated in *Eucalyptus grandis* (Naidoo *et al.*, 2013), the induced response signalling in woody plants is likely mediated by signalling molecules that may be at least partly different from those of herbaceous systems, and in ways that are more complex (Eyles *et al.*, 2010; Zhang *et al.*, 2010). For example, in Norway spruce (*Picea abies*), white-rot fungus (*Heterobasidion parviporum*) infection leads to the parallel induction of both SA and JA pathways (Arnerup *et al.*, 2011), exogenously applied JAs can enhance pathogen resistance (Kozlowski *et al.*, 1999) and exogenously applied SA can increase resistance against *Ips typographus* bark beetles (Krajnc *et al.*, 2011). It is important to note, however, that hormone signalling complexity has also been reported and discussed in model herbaceous plant systems (Kazan & Manners, 2008). Generally, however, the signalling hormones involved in woody plant responses, as well as their interactions (i.e. cross-talk), remain largely unexplored and many aspects of these processes are unknown (Eyles *et al.*, 2010; Zhang *et al.*, 2010). Furthermore, the indirect interactions between herbivorous insects of different feeding guilds via alterations to induced defence responses in woody plants are also largely unknown, especially for conifers.

Stylet-feeding arthropods (i.e. mites and insects) are major conifer pests in both horticultural and forest settings (Cram *et al.*, 2012; Van Driesche *et al.*, 2013) and can be very damaging during outbreaks [e.g. spruce spider mite (*Oligonychus ununguis*); Furniss & Carolin, 1977; Monterey pine needle aphid (*Essigella californica*); Hopmans & Elms, 2013]. Knowledge of mechanisms of induced resistance of conifers to stylet-feeding arthropods is relatively lacking compared with other feeding guilds. Our understanding of how stylet-feeders indirectly interact with co-occurring herbivores (e.g. folivores) of conifers via changes in host quality is also limited. Mattson *et al.* (1989) reported that balsam twig aphid (*Mindarus abietinus*) density was inversely correlated with the survival and development of spruce budworm (*Choristoneura fumiferana*); Grégoire *et al.* (2015) found lower pupal weights in spruce budworm reared on trees that were symptomatic of balsam woolly adelgid (*Adelges piceae*) infestation. In the latter study, it was hypothesized that this relationship reflected decreased foliar quality, although clear relationships between specific adelgid symptoms, foliar secondary metabolites and larval performance could not be detected (Grégoire *et al.*, 2014; Grégoire *et al.*, 2015).

Several studies have investigated the metabolic and physiological effects of the invasive hemlock woolly adelgid (HWA; *Adelges tsugae*) infestation on eastern hemlock (hemlock; *Tsuga canadensis*). There is evidence that HWA feeding causes a hypersensitive-like response in hemlock involving the foliar accumulation of hydrogen peroxide (H_2O_2) (Radville *et al.*, 2011), proline (Gómez *et al.*, 2012) and SA (Schaeffer *et al.*, 2018). Adelgid infestation also increases emissions of methyl salicylate, the volatile methyl ester of SA (Pezet *et al.*, 2013; Pezet & Elkinton, 2014). These physiological effects indicate that HWA infestation induces a hypersensitive-like, SA-linked response in the foliage of this conifer, and this reaction may indirectly affect other herbivores by interfering with typical hormonal responses and induced defences in hemlock (Kroes *et al.*, 2015).

We report the results of research evaluating the ability of HWA to interfere with standard induced defence signalling and expression [as tested by applying methyl jasmonate (MeJA) to plants with and without HWA] and assessing the plant-mediated impact of these treatments on a native folivore, hemlock looper (looper; *Lambdina fiscellaria*). The present study aimed (i) to assess the impact of both SA-linked defences via HWA infestation and JA-linked defences via MeJA application on the performance of a folivore and (ii) to determine whether HWA infestation alters the expression of JA-linked defences and affects the negative impacts of JA-linked defence induction on folivores. We hypothesized that JA-linked responses are more appropriate anti-folivore defences than SA-linked responses and also that the presence of HWA would attenuate the negative effects of JA-linked responses on looper larvae and on the expression of JA-linked defences, presumably as a result of hormone signalling interference.

Materials and methods

Study system

Hemlock is a structurally-dominant and ecologically-important conifer endemic to eastern North America, a 'foundational species' that creates unique and critical habitat for many terrestrial and aquatic species (Snyder *et al.*, 2002; Ellison *et al.*, 2005; Orwig *et al.*, 2008). Hemlock woolly adelgid is an invasive stylet-feeding insect introduced to Virginia in the 1950s (Havill *et al.*, 2006). The invasion of eastern North America by HWA has caused widespread mortality of both eastern and Carolina hemlock (*Tsuga caroliniana*) and threatens to extirpate these species from their native range. The life cycles of HWA specifically, and Adelgidae generally, are described elsewhere (McClure, 1989; Havill & Foottit, 2007). Briefly, HWA is bivoltine, with a holocyclic lifecycle in its native range but an obligate parthenogenetic lifecycle in its introduced range. Although the first-instar 'crawler' phase can move along branches or be passively dispersed between trees (McClure, 1990), adults are sessile, settling and feeding at the base of needles on xylem ray parenchyma cells (Young *et al.*, 1995). Conversely, hemlock looper is native to eastern North America and feeds on many tree species, including eastern and Carolina hemlock (Wilson *et al.*, 2016). This insect has been linked to the mid-Holocene decline of hemlocks in the northeastern U.S.A. (Foster *et al.*, 2006) and widespread defoliation events in Maine in the early 1990s and eastern Canada in the 2000s (Wilson *et al.*, 2016). Larval emergence occurs in the late spring and is timed to coincide with bud burst and the production of new foliage of its conifer hosts (Butt *et al.*, 2010). Late-instar larvae are, however, capable of feeding on older growth (Carroll, 1999). At outbreak densities, the feeding activity of late-instar larvae can cause rapid needle loss and kill mature trees within 2 years (Alfaro *et al.*, 1999). These two herbivores co-occur in the northern portion of the HWA-invaded range and in the southern portion of the native range of the looper (Wilson *et al.*, 2016).

Experimental approach

Approximately 300 hemlock plants were purchased in the spring of 2015 as saplings (0.8–1.0 m in height) from Van Pines Nursery (West Olive, Michigan; derived from seed collected in Pennsylvania). All plants were previously herbivore-free and had not been treated with insecticides. Potted plants (7.6 L per 2 gallon pot size) were placed outside under shade cloth at The University of Rhode Island (URI) (Kingston, Rhode Island), regularly watered and minimally fertilized (14 : 14 : 14; N : P : K; Scotts Osmocote Controlled Release Fertilizer, Australia). Plants were overwintered outside under winter protection fabric (170 g/yard^2; Griffin Greenhouse Supplies, Tewksbury, Massachusetts).

Half of the hemlocks were assigned randomly to the HWA treatment. Each tree in this treatment was inoculated in late spring of 2015, 2016 and 2017 (timed to coincide with HWA progrediens crawler emergence) using locally-collected (Mt Tom State Reservation, Massachusetts), infested hemlock foliage and standard inoculation protocols (Butin *et al.*, 2007). Each potted plant in the HWA received two branches (approximately 15–20 cm long) with densities ≥ 0.5 ovisacs/cm. Plants were annually infested with progrediens generation crawlers as part of ongoing experimentation at URI and to generate an in-house source of HWA for use in unrelated experiments. Additionally, reports of deleterious impacts of HWA on hemlock have been reported mostly in the context of chronic infestation (Radville *et al.*, 2011; Gómez *et al.*, 2012; Pezet *et al.*, 2013; Pezet & Elkinton, 2014; Schaeffer *et al.*, 2018; Wilson *et al.*, 2018). The other half of the hemlocks were assigned to the control (no HWA) treatment. To control for mechanical disturbance, trees in the control treatment were 'sham inoculated' with HWA-free hemlock foliage when trees in the HWA treatment were inoculated with infested foliage. To ensure that control trees remained free of HWA, both infested and uninfested plants were covered with insect-proof mesh (AG-15 Insect Barrier; Agribon, Johnny's Selected Seeds, Waterville, Maine; 90% light transmission). At the time of experimentation, densities of adult progrediens HWA (with ovisacs) were approximately 0.5 HWA/cm on infested trees and control trees were confirmed HWA-free via visual inspection. No quantitative data on plant growth or condition were taken, although visual inspection showed that infested plants were roughly the same size as uninfested plants but the foliage was not the characteristic bright-green of healthy, uninfested plants, such as those in the uninfested treatment.

After the spring 2017 inoculation, 20 trees in the HWA treatment and 20 trees in the control treatment were assigned randomly to one of two elicitor treatments ($n = 10$ per treatment): JA-induced (via MeJA) or constitutive (carrier solution only). MeJA was first dissolved in a minimal amount of absolute ethanol (approximately 0.5 mL) and then suspended in 0.1% (v/v) Tween 20 carrier solution to produce a 1 mM concentration of MeJA (Sigma; St Louis, Missouri). This resulted in four 10-replicate treatments (40 total plants; used in bioassays and in chemical analyses). The appropriate elicitor solution was applied with an atomizer until plants were saturated once every week; preliminary experimentation determined the elicitor concentration used (C. M. Rigsby, unpublished data). Two rounds of elicitor treatments were applied prior to the use of foliage in the bioassay (detailed below) and three rounds of elicitor treatments were applied during the bioassay. Elicitor applications were never made fewer than 4 days prior to the removal of foliage from plants and placing foliage in jars for the looper feeding bioassay. This was carried out to prevent any direct impact of MeJA on larvae. After five elicitor treatments, two randomly selected branches were removed from each plant, wrapped in aluminum foil and stored at −80 °C for chemical analyses. Needle tissue was later separated from stems, ground in liquid nitrogen, partitioned into tubes (see below) and stored at −30 °C until analysis.

Defence responses

Equipment and reagents. We were interested in how our treatments broadly altered the chemistry and physiology of hemlock and therefore elected to utilize more general analytical methods. Bradford assay dye concentrate was purchased from Bio-Rad (Hercules, California) and polyvinylpolypyrrolidone (PVPP; 25 μm average particle size) was purchased from The Vintner Vault (Paso Robles, California). All other reagents and standards were purchased from Sigma (St Louis, Missouri). Spectrophotometric assays were performed in Greiner UV-Star® 96 well plates (Monroe, North Carolina). Plates were read using a SpectraMAX M2 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, California) in the RI-INBRE facility (URI, Kingston, RI).

Defensive enzymes. To extract native protein, 200 mg tissue was reacted with 1.5 mL of 50 mM NaPO4 (pH 6.8) containing 10% (w/v) PVPP, 5% (w/v) Amberlite XAD4 resin (pre-conditioned) and 1 mM ethylenediaminetetraacetic acid on ice for 20 min and the 10 000 ***g*** supernatant (5 min, 4 °C) was recovered and used as the source of enzymes. The guaiacol-oxidizing ($\varepsilon_{470} = 26.6$/mM/cm) activity of peroxidase (POX) was quantified as described by Cipollini *et al.* (2011). The activities of chitinase (CHI) and lipoxygenase (LOX; $\varepsilon_{234} = 23\,000$/M/cm; modifying to accommodate a 96-well microplate format) were quantified as described by Rigsby *et al.* (2016).

Secondary metabolites and H_2O_2. For soluble phenolic metabolites (total soluble phenolics, hydroxycinnamic acids, flavonoids and proanthocyanidins), 200 mg of tissue was twice-extracted in 0.5 mL of methanol for 24 h and the supernatants were pooled. Total soluble phenolic levels were quantified using the modified Folin–Ciocalteau procedure described by Cipollini *et al.* (2011) against a standard curve of gallic acid. Hydroxycinnamic acids were quantified with Arnow's reagent against as described by St-Pierre *et al.* (2013) against standard curve of chlorogenic acid. Total flavonoids were quantified in accordance with the procedure described by Chang *et al.* (2012) against a standard curve of quercetin. Proanthocyanidin content was estimated in accordance with the acidified butanol method (Engström *et al.*, 2014). The lack of affordable standards and concerns with the use of purified standards in the proanthocyanidin assay (Schofield *et al.*, 2001) required that tissue levels were expressed as A_{550} per g fresh weight. Lastly, methanol soluble terpene levels were quantified using H_2SO_4 as described by Ghorai *et al.* (2012) against a standard curve of linalool.

Tissue pellets left over from the extraction of soluble phenolics were washed twice with methanol and cell wall-bound phenolics were extracted via esterification (de Ascensao & Dubery, 2003) and quantified using the total phenolic content procedure described previously with gallic acid as standard. The tissue pellets were then subjected to the lignin extraction and quantification procedure described by Cipollini *et al.* (2011) using spruce lignin as standard.

Needle H_2O_2 levels were estimated in accordance with the KI procedure described by Rigsby *et al.* (2016) using H_2O_2 as a standard curve.

Looper bioassay

In early spring 2017, we obtained looper eggs from a colony maintained at the Canadian Forest Service's Laurentian Forestry Centre (Québec City, Canada). Movement of the eggs from Canada to the U.S.A., and our subsequent work with them, was covered under Animal and Plant Health Inspection Service (APHIS) permit P526P-14-01875. The eggs were placed on arrival in a growth chamber (LD 16 : 8 h photocycle at 15 °C and 75% relative humidity) and monitored daily for hatching. Upon hatching, a 15-cm stem section was clipped from each of the treated plants and stuck in a moistened piece of floral foam within a 0.8-L Ball Mason jar. Each plant provided all of the foliage for a given jar throughout the experiment and contained both current-year foliage and foliage produced in past years. The APHIS permit necessary to work with these larvae required that they be contained in a biological control facility, whereas the potted plants used in these experiments were too large to bring into the facility and be placed in environmental chambers. This necessitated the use of clipped foliage in jars rather than larvae being directly placed on plants. Larvae were assigned randomly to jars as they hatched until each jar contained six looper larvae. Each jar was covered with a fine white mesh (0.5 mm; nylon) to allow ventilation but prevent escape. Jars were kept in the growth chamber, changing their position daily within the growth chamber to account for possible microclimatic differences. Each jar was cleaned weekly by adding a new stem section, replacing the floral foam and removing all waste from the jar. Foliage was never placed into jars within 48 h of being sprayed with elicitor.

We conducted weekly survival assays by removing all foliage and floral foam from the jar and transferring living larvae into clean jars with new foliage and floral foam. Larvae were monitored until pupation, at which point the date of pupation was noted and the pupa were weighed. Data on the six looper larvae per jar were averaged to generate a per-jar mean for each of the 40 replicates.

Statistical analysis

Plant chemical and physiological parameters were analyzed via a two-way analysis of variance (ANOVA) with HWA and MeJA application, as well as their interaction, as predictors. If a significant interaction was found, Tukey's test was used to separate means. For the bioassay experiment, statistical treatment with respect to looper survival, pupal weight and time to pupation was similar to that described by Wilson *et al.* (2016). Briefly, data were inspected for normality (Shapiro–Wilk test) and homoscedacity (Bartlett's test) (all response variables satisfied these requirements) and then a repeated measures-ANOVA was used to analyze the effect of HWA and MeJA application, as well as their interaction. The effect of the same predictors on time to pupation and pupal weights was analyzed using a two-way ANOVA. R software was used for all of the analyses (R Development Core Team, 2017).

Results

Hemlock foliar defence responses

Defensive/antioxidant enzyme activities. Adelgid infestation increased the activity of both POX and CHI but not LOX (Table 1). Elicitor application increased the activity of CHI and LOX but not POX (Table 1) and there was no significant HWA × elicitor interaction for any enzyme activity (Table 1).

Metabolites. Adelgid infestation and MeJA application significantly impacted all classes of soluble phenolics (Table 1). HWA and MeJA both tended to have an additive effect on all phenolic categories; the HWA × elicitor interaction was nonsignificant for all of the soluble phenolic classes (Table 1). HWA infestation increased the cell wall-bound phenolic content of foliage, although there was no effect of MeJA or the HWA × elicitor interaction (Table 1). Adelgid-infested plants also contained more lignin and, although there was no main effect of MeJA, there was an interactive effect between HWA infestation and MeJA application on lignin content where MeJA application appeared to attenuate the HWA-caused increase in lignin. Methanol-soluble terpene content of foliage was not influenced by HWA infestation or elicitor treatment, with terpene content remaining constant between treatment combinations ($P > 0.05$ for all) (Table 1). Lastly, needle H_2O_2 content was elevated by HWA infestation and decreased by MeJA, although there was no significant interactive effect. The H_2O_2 content of foliage was highest in the infested-control treatment and lowest in the uninfested-MeJA treatment (Table 1).

Herbivore responses

HWA infestation reduced the survival of looper larvae over time ($F_{1,36} = 5.49$, $P = 0.0196$) (Fig. 1A) and there was a trend (albeit insignificant; $P = 0.0999$) towards HWA increasing pupal weight ($F_{1,36} = 2.86$, $P = 0.0999$) (Fig. 1B). Although MeJA did not affect larval survival ($F_{1,36} = 0.73$, $P = 0.39$) (Fig. 1A), it did decrease weight at pupation ($F_{1,36} = 7.26$, $P = 0.0107$) (Fig. 1B). The HWA × elicitor interaction affected neither larval survival,

Table 1 The effect of hemlock woolly adelgid (HWA) infestation and methyl jasmonate (MeJA) application, as well as their interaction, on enzyme activities and the metabolites that were quantified

Response variable	Uninfested		Infested		HWA-infestation		MeJA-application		Interaction	
Enzyme activities	Control	MeJA	Control	MeJA	$F_{1,36}$	*P*	$F_{1,36}$	*P*	$F_{1,36}$	*P*
Peroxidase (POX)	157.2 (19.2)	182.6 (40.7)	329.4 (67.3)	393.1 (122.1)	6.8	**0.013**	0.4	0.547	0.1	0.795
Chitinase (CHI)	0.22 (0.04)^{b}	0.33 (0.05)^{b}	0.62 (0.10)^{a}	0.86 (0.10)^{a}	33.6	**< 0.001**	5.1	**0.030**	0.7	0.422
Lipoxygenase (LOX)	74.2 (15.3)^{ab}	92.5 (10.1)^{ab}	69.4 (6.9)^{b}	126.3 (20.4)^{a}	1.1	0.313	7.1	**0.011**	1.9	0.179
Metabolites										
Total soluble Phenolics	78.1 (4.8)^{c}	101.8 (3.3)^{b}	99.5 (5.8)^{b}	131.0 (6.4)^{a}	23.6	**< 0.001**	27.9	**< 0.001**	0.6	0.457
Hydroxycinnamic acids	35.8 (1.5)^{b}	54.9 (4.5)^{a}	47.6 (3.9)^{ab}	58.3 (2.6)^{a}	4.3	**0.046**	19.3	**0.001**	1.5	0.232
Flavonoids	50.6 (2.2)^{b}	66.1 (2.0)^{a}	62.1 (3.4)^{a}	70.1 (1.6)^{a}	10.7	**0.002**	24.2	**< 0.001**	2.5	0.124
Proanthocyanidins	0.6 (0.1)^{c}	1.4 (0.1)^{b}	1.2 (0.17)^{b}	1.8 (0.2)^{a}	11.7	**0.002**	22.4	**< 0.001**	0.2	0.650
Cell Wall-bound Phenolics	10.0 (1.5)^{b}	13.4 (1.8)^{ab}	22.1 (6.0)^{a}	18.1 (2.1)^{ab}	6.7	**0.014**	0.0	0.919	1.3	0.258
Lignin	3.8 (0.2)^{b}	4.5 (0.2)^{ab}	4.9 (0.3)^{a}	4.5 (0.2)^{ab}	7.3	**0.011**	0.4	0.532	5.8	**0.021**
Methanol-soluble terpenes	2.8 (0.4)	2.5 (0.1)	3.1 (0.1)	2.8 (0.2)	1.9	0.177	1.8	0.186	0.0	0.918
H_2O_2	65.7 (11.9)^{ab}	15.2 (3.3)^{b}	133.6 (39.1)^{a}	39.9 (13.6)^{b}	4.3	**0.047**	13.1	**0.001**	1.2	0.285

F- and *P*-values (significant values are indicated in bold) are the results of a two-way analysis of variance using HWA-infestation and MeJA-application, as well as their interaction, as predictor variables. Different lowercase letters indicate significant differences between treatment combinations according to a post-hoc Tukey's test, and an absence of letters indicates no significant treatment differences.

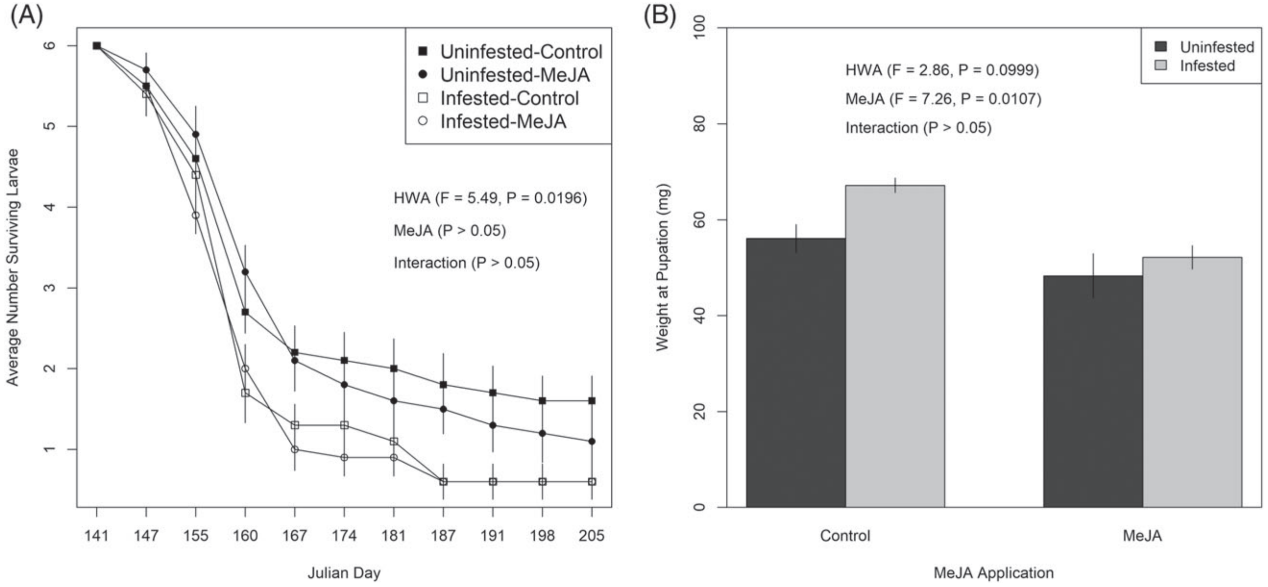

Figure 1 Response of hemlock looper to hemlock woolly adelgid (HWA) infestation and methyl jasmonate (MeJA) application. (A) Mean ± SE number surviving looper larvae throughout the feeding time on foliage of plants from the four treatments. (B) Mean ± SE pupal weight in mg of hemlock looper larvae fed foliage of plants from the four treatments.

nor pupal weights ($P > 0.05$). Time to pupation was not affected by any predictor variable ($P > 0.05$).

Discussion

We found that changes in hemlock physiology associated with an invasive herbivore and with elicitor application affected both secondary chemistry and the response of a native herbivore. Although our initial hypothesis of HWA/MeJA (i.e. SA/JA) antagonism was generally not supported, the physiological responses of hemlock that we observed appear to be partly mediated by both SA and JA pathways. Such antagonistic responses are important because plants must often respond to simultaneous or sequential challenges (Ponzio *et al.*, 2013). Moreover, our results are consistent with the ability of stylet-feeding insects to manipulate plant physiology via induced defences linked to this cross-talk in ways that can dramatically alter host quality for other herbivores (Inbar *et al.*, 1999). Historically, there has been little research specifically addressing JA–SA cross-talk and indirect herbivore effects in woody plants. The hemlock–HWA system provides an excellent model for better understanding these indirect interactions because chronic HWA infestation results in SA induction and a hypersensitive-like response in its host (Radville *et al.*, 2011; Gómez *et al.*, 2012; Pezet *et al.*, 2013; Pezet & Elkinton, 2014; Schaeffer *et al.*, 2018). We had expected that both HWA infestation (SA induction) and MeJA (JA induction) would induce changes in hemlock chemistry and physiology and would also affect looper performance, whereas simultaneous challenge would result in hormonal signalling interference that compromises the induction and expression of appropriate anti-folivore defences, ultimately positively influencing looper larvae.

We found certain defensive traits to be distinctly elicited by one treatment, some of which were predictable. For example, LOX activity was positively affected by MeJA application and HWA infestation had a positive impact on H_2O_2 accumulation. These traits are associated with their respective signalling responses because LOX has a direct role in JA synthesis (Beckers & Spoel, 2006) and H_2O_2 accumulation is associated with SA signalling both upstream and downstream of SA (Herrera-Vásquez *et al.*, 2015). Intriguingly, POX activity and cell wall-bound phenolic and lignin accumulation were positively affected only by HWA infestation. Peroxidases use H_2O_2 as a co-substrate to polymerize phenolics and monolignols, which serve to scavenge H_2O_2 (Tenhaken, 2014). The extent to which the HWA-mediated increase in POX activity, cell wall-bound phenolic and lignin accumulation is an antioxidant response to H_2O_2 accumulation or an SA-linked anti-herbivore response remains to be determined. We also found, however, that certain defensive traits were not strictly regulated by one induction treatment or the other, with these responses appearing to be additive rather than antagonistic (i.e. CHI activity and soluble phenolics). One defensive trait (methanol-soluble terpene content) was not influenced by either treatment, although this is not necessarily surprising because it has been shown that conifers may not accumulate foliar terpenes after herbivore attack (e.g. Litvak & Monson, 1998). Additionally, the use of methanol to extract terpenes, as per this assay method (Ghorai *et al.*, 2012), may limit the interpretation of the results of the assay because methanol is a relatively meager solvent for nonpolar terpene species.

One of the more important and interesting results of the present study, confirming the findings of previous research (Radville *et al.*, 2011), is not only that hemlock accumulates H_2O_2 when infested with HWA, but also that H_2O_2 did not accumulate when plants were sprayed with MeJA. Hydrogen peroxide has a variety of functions in plants in addition to being a co-substrate for POX enzymes (Cheeseman, 2007), including roles in stress response-signalling (Orozco-Cárdenas *et al.*, 2001; Morkunas *et al.*, 2011; Petrov & Van Breusegem, 2012). For example, H_2O_2 accumulation resulted in the identification of 82 H_2O_2-responsive proteins in the leaves of seedling hybrid poplars (*Populus simonii* × *Populus nigra*) (Yu *et al.*, 2017). Hydrogen peroxide has also been shown to both amplify and antagonize SA signalling/accumulation (Peleg-Grossman *et al.*, 2010; Petrov & Van Breusegem, 2012). The ultimate implications and impacts of H_2O_2 accumulation in hemlock foliage remain unknown, although these are likely consequential because H_2O_2 accumulation could have any of the described effects in hemlock or others. Furthermore, the interaction between H_2O_2 and JA, and specifically the fact that JA pathway activation (via MeJA application) results in a reduction in H_2O_2 levels regardless of HWA infestation, suggests that antioxidant mechanisms are part of JA pathway elicitation.

The effects of our treatments on hemlock foliar defences and the ultimate impacts on looper larvae were mixed. Our hypothesis that JA-linked responses are appropriate anti-folivore defences was supported; our hypothesis that HWA infestation would interfere with standard anti-folivore (i.e. JA) induced defence signalling and would attenuate the negative effects of JA-linked responses on looper was not supported. For example, MeJA application reduced looper pupal weights but did not affect looper survival, whereas HWA did not significantly impact pupal weights or larval survival. This suggests that induced defence signalling is more nuanced than simple JA–SA antagonism in hemlock and also that both hormones likely have roles. The notion that extensive JA–SA cross-talk exists in plant biotic stress response signalling is not novel (Smith *et al.*, 2009), although these findings highlight the complex nature of this cross-talk and how additional complexity can be introduced when plants are attacked by multiple herbivores (Nguyen *et al.*, 2016).

In the present study, we demonstrated that HWA induces defence responses involving phenolic metabolites and antioxidant/defensive proteins; these responses are not necessarily the same in MeJA-induced plants; and some responses were additive (e.g. phenolics). The treatment-associated physiological effects on hemlock foliage had mixed effects on looper larval performance, where survival was negatively impacted by HWA infestation and MeJA application negatively impacted pupal weight. Our results only partly supported our initial hypotheses that JA-linked responses are more appropriate anti-folivore defences, and that HWA infestation would benefit folivores by interfering with standard anti-folivore (i.e. JA-linked) hormonal signalling. It is possible that the infestation level of our plants (0.5 HWA/cm), although ecologically relevant, may not have been sufficient to result in our hypothesized effects. The results of the present study not only illustrate how HWA-mediated plant defence induction can alter the suitability of this conifer for other co-occurring herbivores, but also emphasize the need to further study multistress interactions and physiological antagonism in conifers.

Acknowledgements

We thank two anonymous reviewers for their helpful comments that substantially improved the clarity of the work. We also thank A. Baranowski, R. Casagrande and L. Tewksbury for their assistance with looper bioassays; K. Andrews and A. Bach for their assistance with RI-INBRE facility equipment; and A. Labrecque of the Laurentian Forestry Centre for providing *L. fiscellaria* eggs.

Funding

This project was funded by National Science Foundation grants NSF-DEB 1256826 to C. Orians and NSF-DEB 1256769 to E. Preisser and C. Thornber; C. Rigsby was supported by USDA McIntire-Stennis RI0017-MS979 and PA Department of Conservation and Natural Resources DCNR 2016-001-HWA-U.

References

Alfaro, R.I., Taylor, S., Brown, G. & Wegwitz, E. (1999) Tree mortality caused by the western hemlock looper in landscapes of central British Columbia. *Forest Ecology and Management*, **124**, 285–291.

Arnerup, J., Lind, M., Olson, Å., Stenlid, J., Elfstrand, M. & Näsholm, T. (2011) The pathogenic white-rot fungus *Heterobasidion parviporum* triggers non-specific defence responses in the bark of Norway spruce. *Tree Physiology*, **31**, 1262–1272.

de Ascensao, A.R.F.D.C. & Dubery, I.A. (2003) Soluble and wall-bound phenolics and phenolic polymers in *Musa acuminata* roots exposed to elicitors from *Fusarium oxysporum* f.sp. *cubense*. *Phytochemistry*, **63**, 679–686.

Baldwin, I.T. (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proceedings of the National Academy of Sciences*, **95**, 8113–8118.

Beckers, G.J. & Spoel, S.H. (2006) Fine-tuning plant defence signalling: salicylate versus Jasmonate. *Plant Biology*, **8**, 1–10.

Bonello, P., Gordon, T., Herms, D., Wood, D. & Erbilgin, N. (2006) Nature and ecological implications of pathogen-induced systemic resistance in conifers: a novel hypothesis. *Physiological and Molecular Plant Pathology*, **68**, 95–104.

Butin, E., Preisser, E. & Elkinton, J. (2007) Factors affecting settlement rate of the hemlock woolly adelgid, *Adelges tsugae*, on eastern hemlock, *Tsuga canadensis*. *Agricultural and Forest Entomology*, **9**, 215–219.

Butt, C., Quiring, D., Hébert, C., Delisle, J., Berthiaume, R., Bauce, E. & Royer, L. (2010) Influence of balsam fir (*Abies balsamea*) budburst phenology on hemlock looper (*Lambdina fiscellaria*). *Entomologia Experimentalis et Applicata*, **134**, 220–226.

Carroll, A.L. (1999) Physiological adaptation to temporal variation in conifer foliage by a caterpillar. *The Canadian Entomologist*, **131**, 659–669.

Chang, C.L., Lin, C.S. & Lai, G.H. (2012) Phytochemical characteristics, free radical scavenging activities, and neuroprotection of five medicinal plant extracts. *Evidence-based Complementary and Alternative Medicine*, **2012**, 984295.

Cheeseman, J. (2007) Hydrogen peroxide and plant stress: a challenging relationship. *Plant Stress*, **1**, 4–15.

Cipollini, D., Enright, S., Traw, M.B. & Bergelson, J. (2004) Salicylic acid inhibits jasmonic acid-induced resistance of *Arabidopsis thaliana* to *Spodoptera exigua*. *Molecular Ecology*, **13**, 1643–1653.

Cipollini, D., Wang, Q., Whitehill, J.G.A., Powell, J.R., Bonello, P. & Herms, D.A. (2011) Distinguishing defensive characteristics in the phloem of ash species resistant and susceptible to emerald ash borer. *Journal of Chemical Ecology*, **37**, 450–459.

Cram, M.M., Frank, M.S. & Mallams, K.M. (2012) *Forest Nursery Pests*. Agriculture Handbook 680 rev. 2012. U. S. Department of Agriculture, Forest Service, Washington D.C.

Ellison, A., Bank, M.S., Clinton, B.D. *et al.* (2005) Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. *Frontiers in Ecology and the Environment*, **3**, 479–486.

Engström, M.T., Palijarvi, M., Fryganas, C., Grabber, J.H., Mueller-Harvey, I. & Salminen, J.P. (2014) Rapid qualitative and quantitative analyses of proanthocyanidin oligomers and polymers by UPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, **62**, 3390–3399.

Eyles, A., Bonello, P., Ganley, R. & Mohammed, C. (2010) Induced resistance to pests and pathogens in trees. *New Phytologist*, **185**, 893–908.

Foster, D., Oswald, W., Faison, E., Doughty, E. & Hansen, B. (2006) A climatic driver for abrupt mid-Holocene vegetation dynamics and the hemlock decline in New England. *Ecology*, **87**, 2959–2966.

Furniss, R.L. & Carolin, V.M. (1977) *Western Forest Insects*. U.S. Government Printing Office, Washington, District of Columbia.

Ghorai, N., Chakraborty, S., Gucchait, S., Saha, S.K. & Biswas, S. (2012) Estimation of total terpenoids concentration in plant tissues using a monoterpene, linalool as standard reagent. *Nature Protocol Exchange*. https://doi.org/10.1038/protex.2012.055.

Gómez, S., Orians, C. & Preisser, E. (2012) Exotic herbivores on a shared native host: tissue quality after individual, simultaneous, and sequential attack. *Oecologia*, **169**, 1015–1024.

Grégoire, D.M., Quiring, D.T., Royer, L. & Bauce, É. (2014) Relationship between an exotic phloem feeder and balsam fir (*Abies balsamea* L. (Mill.)) foliar chemistry: implications for two native defoliators. *Arthropod-Plant Interactions*, **8**, 361–371.

Grégoire, D.M., Quiring, D.T., Royer, L., Heard, S.B. & Bauce, É. (2015) Indirect host-mediated effects of an exotic phloem-sap feeder on a native defoliator of balsam fir. *Forest Ecology and Management*, **341**, 1–7.

Havill, N. & Foottit, R. (2007) Biology and evolution of Adelgidae. *Annual Review of Entomology*, **52**, 325–349.

Havill, N., Montgomery, M., Yu, G., Shiyake, S. & Caccone, A. (2006) Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. *Annals of the Entomological Society of America*, **99**, 195–203.

Herrera-Vásquez, A., Salinas, P. & Holuigue, L. (2015) Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. *Frontiers in Plant Science*, **6**, 171.

Hopmans, P. & Elms, S.R. (2013) Impact of defoliation by *Essigella californica* on the growth of mature Pinus radiata and response to N, P and S fertilizer. *Forest Ecology and Management*, **289**, 190–200.

Inbar, M., Doostdar, H. & Mayer, R.T. (1999) Effects of sessile whitefly nymphs (Homoptera: Aleyrodidae) on leaf-chewing larvae (Lepidoptera: Noctuidae). *Environmental Entomology*, **28**, 353–357.

Kazan, K. & Manners, J.M. (2008) Jasmonate signaling: toward an integrated view. *Plant Physiology*, **146**, 1459–1468.

Kozlowski, G., Buchala, A. & Metraux, J.-P. (1999) Methyl jasmonate protects Norway spruce [*Picea abies* (L.) Karst.] seedlings against *Pythium ultimum* Trow. *Physiological and Molecular Plant Pathology*, **55**, 53–58.

Krajnc, A.U., Kristl, J. & Ivancic, A. (2011) Application of salicylic acid induces antioxidant defense responses in the phloem of *Picea abies* and inhibits colonization by *Ips typographus*. *Forest Ecology and Management*, **261**, 416–426.

Kroes, A., van Loon, J.J. & Dicke, M. (2015) Density-dependent interference of aphids with caterpillar-induced defenses in *Arabidopsis*: involvement of phytohormones and transcription factors. *Plant and Cell Physiology*, **56**, 98–106.

Krokene, P. (2015) Conifer defense and resistance to bark beetles. *Bark Beetles Biology and Ecology of Native and Invasive Species* (ed. by F. E. Vega and R. W. Hofstetter), pp. 177–207. Academic Press, San Diego, California.

Litvak, M.E. & Monson, R.K. (1998) Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. *Oecologia*, **114**, 531–540.

Mattson, W.J., Haack, R., Lawrence, R.K. & Herms, D.A. (1989) Do balsam twig aphids (Homoptera: Aphididae) lower tree susceptibility to spruce budworm? *The Canadian Entomologist*, **121**, 93–103.

McClure, M. (1989) Evidence of a polymorphic life cycle in the hemlock woolly adelgid *Adelges tsugae* (Homoptera: Adelgidae). *Annals of the Entomological Society of America*, **82**, 50–54.

McClure, M. (1990) Role of wind, birds, deer, and humans in the dispersal of hemlock woolly adelgid (Homoptera, Adelgidae). *Environmental Entomology*, **19**, 36–43.

Morkunas, I., Mai, V.C. & Gabryś, B. (2011) Phytohormonal signaling in plant responses to aphid feeding. *Acta Physiologiae Plantarum*, **33**, 2057–2073.

Naidoo, R., Ferreira, L., Berger, D.K., Myburg, A.A. & Naidoo, S. (2013) The identification and differential expression of *Eucalyptus grandis* pathogenesis-related genes in response to salicylic acid and methyl jasmonate. *Frontiers in Plant Science*, **4**, 43.

Nguyen, D., Rieu, I., Mariani, C. & van Dam, N.M. (2016) How plants handle multiple stresses: hormonal interactions underlying responses to abiotic stress and insect herbivory. *Plant Molecular Biology*, **91**, 727–740.

O'Donnell, P.J., Schmelz, E., Block, A., Miersch, O., Wasternack, C., Jones, J.B. & Klee, H.J. (2003) Multiple hormones act sequentially to mediate a susceptible tomato pathogen defense response. *Plant Physiology*, **133**, 1181–1189.

Orozco-Cárdenas, M.L., Narváez-Vásquez, J. & Ryan, C.A. (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *The Plant Cell*, **13**, 179–191.

Orwig, D., Cobb, R., D'Amato, A., Kizlinski, M. & Foster, D. (2008) Multi-year ecosystem response to hemlock woolly adelgid infestation in southern New England forests. *Canadian Journal of Forest Research*, **38**, 834–843.

Peleg-Grossman, S., Melamed-Book, N., Cohen, G. & Levine, A. (2010) Cytoplasmic H_2O_2 prevents translocation of NPR1 to the nucleus and inhibits the induction of PR genes in *Arabidopsis*. *Plant Signaling and Behavior*, **5**, 1401–1406.

Petrov, V.D. & Van Breusegem, F. (2012) Hydrogen peroxide-a central hub for information flow in plant cells. *AoB Plants*, **2012**, pls014.

Pezet, J. & Elkinton, J.S. (2014) Hemlock woolly adelgid (Hemiptera: Adelgidae) induces twig volatiles of eastern hemlock in a forest setting. *Environmental Entomology*, **43**, 1275–1285.

Pezet, J., Elkinton, J.S., Gomez, S., McKenzie, E.A., Lavine, M. & Preisser, E.L. (2013) Hemlock woolly adelgid and elongate hemlock scale induce changes in foliar and twig volatiles of eastern hemlock. *Journal of Chemical Ecology*, **39**, 1090–1100.

Ponzio, C., Gols, R., Pieterse, C.M.J., Dicke, M. & Bennett, A. (2013) Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. *Functional Ecology*, **27**, 587–598.

Preston, C.A., Lewandowski, C., Enyedi, A.J. & Baldwin, I.T. (1999) Tobacco mosaic virus inoculation inhibits wound-induced jasmonic acid-mediated responses within but not between plants. *Planta*, **209**, 87–95.

R Development Core Team (2017) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Austria.

Radville, L., Chaves, A. & Preisser, E.L. (2011) Variation in plant defense against invasive herbivores: evidence for a hypersensitive response in eastern hemlocks (*Tsuga canadensis*). *Journal of Chemical Ecology*, **37**, 592–597.

Ralph, S.G., Yueh, H., Friedmann, M. *et al.* (2006) Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant, Cell and Environment*, **29**, 1545–1570.

Rigsby, C.M., Herms, D.A., Bonello, P. & Cipollini, D. (2016) Higher activities of defense-associated enzymes may contribute to greater resistance of Manchurian ash to emerald ash borer than a closely related and susceptible congener. *Journal of Chemical Ecology*, **42**, 782–792.

Schaeffer, R.N., Wang, Z., Thornber, C.S., Preisser, E.L. & Orians, C.M. (2018) Two invasive herbivores on a shared host: patterns and consequences of phytohormone induction. *Oecologia*, **186**, 973–982.

Schiebe, C., Hammerbacher, A., Birgersson, G. *et al.* (2012) Inducibility of chemical defenses in Norway spruce bark is correlated with unsuccessful mass attacks by the spruce bark beetle. *Oecologia*, **170**, 183–198.

Schofield, P., Mbugua, D.M. & Pell, A.N. (2001) Analysis of condensed tannins A review. *Animal Feed Science and Technology*, **91**, 21–40.

Smith, J.L., De Moraes, C.M. & Mescher, M.C. (2009) Jasmonate- and salicylate-mediated plant defense responses to insect herbivores, pathogens and parasitic plants. *Pest Management Science*, **65**, 497–503.

Snyder, C., Young, J., Lemarié, D. & Smith, D. (2002) Influence of eastern hemlock (*Tsuga canadensis*) forests on aquatic invertebrate assemblages in headwater streams. *Canadian Journal of Fisheries and Aquatic Sciences*, **59**, 262–275.

Soler, R., Badenes-Pérez, F.R., Broekgaarden, C., Zheng, S.-J., David, A., Boland, W. & Dicke, M. (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. *Functional Ecology*, **26**, 156–166.

St-Pierre, F., Achim, A. & Stevanovic, T. (2013) Composition of ethanolic extracts of wood and bark from *Acer saccharum* and *Betula alleghaniensis* trees of different vigor classes. *Industrial Crops and Products*, **41**, 179–187.

Tenhaken, R. (2014) Cell wall remodeling under abiotic stress. *Frontiers in Plant Science*, **5**, 771.

Thaler, J.S., Humphrey, P.T. & Whiteman, N.K. (2012) Evolution of jasmonate and salicylate signal crosstalk. *Tredns in Plant Science*, **17**, 260–270.

Van Driesche, R.G., LaForest, J.H., Bargeron, C.T., Reardon, R.C. & Herlihy, M. (2013) *Forest Pest Insects in North America: A Photographic Guide*. USDA Forest Service, Morgantown, West Virginia.

Wilson, C.M., Vendettuoli, J.F., Orwig, D.A. & Preisser, E.L. (2016) Impact of an invasive insect and plant defense on a native forest defoliator. *Insects*, **7**, 45.

Wilson, C.M., Schaeffer, R.N., Hickin, M.L. *et al.* (2018) Chronic impacts of invasive herbivores on a foundational forest species: a whole-tree perspective. *Ecology*, **99**, 1783–1791.

Young, R., Shields, K. & Berlyn, G. (1995) Hemlock woolly adelgid (Homoptera: Adelgidae): stylet bundle insertion and feeding sites. *Annals of the Entomological Society of America*, **88**, 827–835.

Yu, J., Jin, X., Sun, X. *et al.* (2017) Hydrogen peroxide response in leaves of Poplar (*Populus simonii* x *Populus nigra*) revealed from physiological and proteomic analyses. *International Journal of Molecular Sciences*, **18**, 2085.

Zhang, J., Du, X., Wang, Q. *et al.* (2010) Expression of pathogenesis related genes in response to salicylic acid, methyl jasmonate and 1-aminocyclopropane-1-carboxylic acid in *Malus hupehensis* (Pamp.) Rehd. *BMC Research Notes*, **3**, 208–208.

Accepted 12 January 2019