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Effect of different headspace concentrations of bornyl acetate on fecundity of green peach aphid and balsam woolly adelgid

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ABSTRACT

Balsam woolly adelgid (*Adelges piceae*) (Hemiptera: Adelgidae) (BWA) is an exotic pest introduced from Europe to North America in the early 1900s. Subsequent introductions and spread have enabled this pest to infest native Fraser fir stands in the Southern Appalachians and become a troublesome pest for the region's Christmas tree industry. Means to study its fecundity and control it are consequently of high importance. Headspace solid phase micro-extraction coupled with gas chromatography and mass spectrometry were used to compare chemical differences in stem tissue between a resistant species, Veitch fir (*Abies veitchii*) and the susceptible Fraser fir (*Abies fraseri*). Comparisons demonstrated that bornyl acetate (BA), a terpenoid, was qualitatively more abundant in resistant Veitch fir than Fraser fir. Varying headspace concentrations of BA were tested to ascertain any biological impacts on egg eclosion of BWA, as well as fecundity of green peach aphid (*Myzus persicae*) (GPA), an insect serving as a proxy. Varying concentrations of BA and a known number of adelgid eggs did not indicate any impact of concentration on egg eclosion success. However, defoliated Veitch fir branches in treatment jars produced a significant negative impact on BWA eclosion success. Implications of these findings are discussed.

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Introduction

Balsam woolly adelgid (*Adelges piceae* Ratz.) (Hempitera: Adelgidae) (BWA) is an exotic invasive insect pest, introduced to the Eastern United States in the early 1900s, and reaching the Southern Appalachian Mountains by 1955 (Kotinsky 1916; Speers 1958). It is a pest of many different species within the genus *Abies* Mill. worldwide (Mitchell 1966). Within its native range of Northern Europe, it infests European silver fir (*Abies alba* Miller), but does not significantly damage or have a major effect on the life cycle of that tree species. Within the Southern Appalachians of the United States, BWA readily infests Fraser fir (*Abies fraseri* (Pursh) Poir) and has a significant effect on growth and survival of this endemic species (Hain et al. 1991). In North America, the BWA life cycle is anholocyclic, meaning that it does not change hosts and reproduces via parthenogenesis only. It is closely related to a species of holocyclic adelgid, *A. nordmannianae* (Eckstein), which is cyclically parthenogenetic, with sexual generations and asexual generations (holocyclic) while also practicing host alternation (Havill et al. 2007).

Resistance screening for BWA across a wide range of members of the *Abies* genus shows that Fraser fir is among the most susceptible species of fir to BWA, while Veitch fir (*Abies veitchii* Lindley), a Japanese species, is among the least susceptible to damage from BWA (Mitchell 1966). Resistance to pests in plants is generally defined within three broad categories: antixenosis, tolerance, and antibiosis (Painter 1958). Resistance to BWA can be placed into multiple categories depending on the host species, which is the most important factor determining adelgid populations (Amman 1970). Some researchers have suggested that juvabione, a compound that mimics juvenile hormones in insects, increased after infestation by BWA within Fraser fir, but were ultimately unsuccessful in finding significant differences in concentrations of infested tissues (Fowler et al. 2001). The formation of secondary periderm around infestation sites may also be a resistance mechanism, protecting the underlying bark, as well as keeping the wound response of the tree from spreading (Mullick 1975). Additional resistance mechanisms to BWA that have been suggested include adaptation to handle drought stress (Mitchell 1966), as well as bark thickness, which displays genetic variability within fir species (Hollingsworth & Hain 1992). In addition to these investigations, predatory species introductions as biocontrol agents for BWA have largely been unsuccessful (Mitchell & Wright 1967).

While these potential mechanisms have been studied mostly by assessing Fraser fir and susceptibility based on intraspecific variation, few have attempted to address differences of an interspecific nature. Utilizing tandem gas chromatography and mass spectrometry (GC/MS) analyses performed on the highly resistant Veitch fir and susceptible Fraser fir, one pronounced difference discovered was the qualitative abundance of bornyl acetate (BA), a terpenoid, present in Veitch fir, compared to lower levels in Fraser fir

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(Bucholz 2015) (Figure 1). While there are many potential target terpenes, as indicated in the subtraction chromatogram in Figure 1, BA stood out as a good starting point for our investigations. BA, found commonly in gymnosperms and angiosperms, is a monoterpene especially present in coniferous trees (Raffa & Powell 2004). Studies have shown a difference in its effect on various insect species. BA reduced larval growth and survival to adult stage of the western spruce budworm (*Choristoneura occidentalis* Freeman) (Lepidoptera: Tortricidae) when combined with high and low levels of nitrogen, administered in an agar diet (Cates et al. 1987). Others have found it to be one of the few monoterpenes isolated from Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) needles that reduced survival and growth rate of multiple, geographically different, populations of western spruce budworm (Zou & Cates 1997). Similarly, BA fed to spruce budworm (*Choristoneura fumiferana* Clemens) at levels higher than those found in balsam fir (*Abies balsamea* (L.) Mill.) needles reduced larval growth (Kumbasli & Bauce 2013). BA has been implicated as a potential management technique for control of stored food and house dust mites (Lee et al. 2009), but has also been implicated as a feeding attractant for certain fly larvae species on carrot root (Ryan & Guerin 1982). BA, when fed to four separate species of tussock moths (Lepidoptera: Lymantriidae), did not yield any changes in larval growth rate, consumption rate, or development times (Raffa & Powell 2004). The reproductive success of two scale insect species (Hemiptera: Dispididae) was evaluated on *Tsuga canadensis* (L.) Carrière and *Tsuga sieboldii* Carrière. On *T. canadensis* the number of eggs was significantly less than that laid by the species on *T. sieboldii*, and one of the significant differences between terpene content between the two species was BA, among other chemical variations (McClure & Hare 1984). Similarly, Hemlock Woolly Adelgid (HWA) (*Adelges tsugae* Annand) (Hemiptera: Adelgidae) has been shown to influence terpene production in infested hemlock (Broeckling & Salom 2003). Also, hemlock species susceptible to HWA produce greater amounts of BA compared to resistant species (Lagalante & Montgomery 2003), a result counter results with BWA (Bucholz 2015). These studies do not provide definitive proof of the effect BA has on various insect species, but they do suggest that there is an effect on certain insect species and not on others, and whether it alone has potential negative/positive effects on various insect species remains in question (Kumbasli & Bauce 2013). With that in mind, BA became a target for these investigations into possible resistance mechanisms for *Abies* spp. to BWA.

Laboratory rearing of BWA is challenging because no artificial media have been developed and eggs are only available once a year from natural populations. We chose to develop our experimental protocol on a model species, the green peach aphid (*Myzus persicae* Sulzer) (Homoptera: Aphididae) (GPA) while the natural BWA populations were in diapause and eggs were not available. GPA is a common agricultural pest, capable of vectoring many different plant pathogens (Van Emden & Harrington 2007). GPA, like closely related adelgid species and many other aphid species, exhibits cyclical parthenogenesis, enabling it to alternate between sexual and asexual generations, an evolutionary advantage that enables GPA to both produce large populations while also enabling it to maintain genetic diversity (Poupoulidou et al. 2006). Although there are differences between the vivipary of GPA and the oviposition of BWA, we chose to develop our protocol on GPA because of its easy rearing capacity and as an interesting comparison to BWA (Gavkare & Gupta 2013). After the protocol was successfully evaluated with GPA and natural BWA populations began ovipositing, collections were made to duplicate the GPA experiments using BWA.

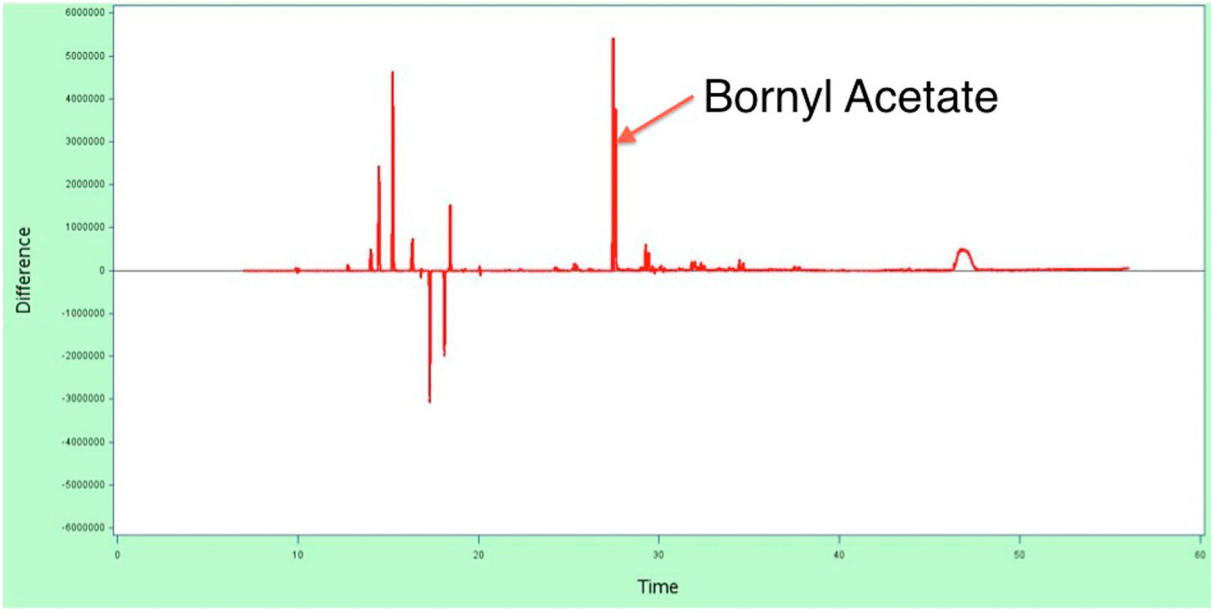

Figure 1. Subtraction chromatogram of Veitch vs. Fraser fir acetone-soluble chemicals from branch stem tissue. Compounds above the 0 line are of greater abundance in Veitch fir, and below, are of greater abundance in Fraser fir.

In preliminary trials, the BA concentration of Fraser fir cuttings was not altered when live cuttings were placed in aqueous BA solutions (Bucholz 2015). Also pure BA (far exceeding natural levels in firs) had a negative impact on egg survival, but also had a phytotoxic effect on the Fraser fir cuttings. Previous experimentation also showed volatiles from both Veitch and Fraser fir cuttings in an open environment had no effect on egg eclosion success when a known number of eggs were placed on each cutting within a Petri dish (Bucholz 2015). Therefore, in order to better manipulate BA concentration, we developed a simple protocol to vary its volatile concentration in the headspace of vessels by diluting BA in silicone oil and ascertaining concentration differences by solid phase micro-extraction (SPME) fiber sample collection and GC-MS analysis. The objectives of the investigations reported here were to employ this technique to determine the effect of BA concentrations on vivipary in GPA and egg eclosion in BWA.

The overall goal of this study was to see if a certain target volatile resulted in a negative effect on BWA egg eclosion. As stated previously, GPA was used as a proxy to test both our experimental apparatus, as well as to ascertain if BA resulted in decreased fecundity on another parthenogenetic homopteran. The experiment was repeated using BWA, once populations became available. Based on the literature, we hypothesized a decrease in fecundity as BA concentration increased, compared to controls. We chose BA based on preliminary results of GC/MS analyses of volatile differences between Veitch and Fraser fir as a starting point for investigating how to test biological effects of certain terpenes on BWA. Elucidating which terpenes play a role in BWA resistance would aid future *Abies* tree improvement programs in selecting and breeding for resistance, as well as increase our overall understanding of what determines resistance to BWA.

Methods

Aphid colony establishment

In March 2015, GPA were collected from a NCSU laboratory assessing the effects of GPA on tobacco (*Nicotiana* spp.) and various *Brassica* species. Before placement of GPA-infested plant material into a rearing chamber, a colony-housing unit was built. Twelve *Brassica oleracea* L. plants were placed into six plastic pots (10.16 cm × 10.16 cm × 12.7 cm) (two per pot) and covered by the top of the housing. Once the newly transferred plants were allowed to grow for a week, GPA-infested plant material was placed on top of the plants, and given two weeks for infestation of the new plant material and for the colony to build.

Volatile concentration determination

The GC/MS analyses for these investigations were performed on an Agilent Model 7280A GC system with an attached Agilent Model 5977E mass spectrometer. Conditions for both the GPA experiment and BWA experiment were the same. Oven temperatures started at 40°C for 2 minutes, and ramped at a rate of 8°C per minute up to a final temperature of 224°C after 25 minutes. Sample separation was accomplished using an Agilent HP-5MS-UI Column (30 m, 0.25 mm id, 0.25 micron thickness). Injector temperatures were kept at a constant 250°C. The SPME fiber was inserted into the injector for all 25 minutes of the run, before removal for other run MS was conducted through electron impact. Transfer line temperatures were kept at 250°C, while the MS Quad was kept at 150°C and the source at 230°C. Mass range scanned was between 40.00 and 250.00 m/z. National Institute of Standards and Technology (NIST) libraries were used to identify compounds.

Determination of the experimental conditions required preliminary analysis through GC/MS of many different branch samples from both species as well as different BA concentrations. Samples of Veitch and Fraser fir were collected from Avery County, NC in mid-March 2015. Defoliated samples were placed in 60 mL SPME jars from Cole-Parmer and allowed 2 days to equilibrate. SPME fibers were exposed to the headspace for 2 hours before being thermally desorbed in the GC/MS. Extraction times were 2 hours based on analyses of signal changes accompanying different extraction lengths. Peak areas were matched based on qualitative relative abundance to determine concentrations that roughly simulated what is found in Fraser and Veitch fir (Bucholz 2015). Silicone oil was used as the diluting agent for the BA, because BA is highly water insoluble. Silicone oil was chosen as the solvent as it is relatively inert and would not evaporate through the course of the experiment. Concentrations representing peak areas similar to Veitch and Fraser fir were selected based on SPME analyses. Once approximate concentrations had been developed, leaves from the host plants within the aphid colony were added to the SPME jars. The leaves absorbed some of the BA, and thus weakened the signal, as opposed to having no leaf material in the jars. Small changes were made based on those findings, and five concentrations were developed. Concentrations 2 and 4 represented Fraser and Veitch fir BA signals respectively.

The five concentrations of BA were dilutions. Concentrations 1 through 5 were 1 µL BA in 10 mL oil, 1 µL BA in 2.5 mL oil, 1 µL BA in 1 mL oil, 10 µL BA in 1 mL oil, and 25 µL BA in 1 mL oil, respectively.

GPA experiment

One-hundred microliter of each solution were added to each SPME jar. The jars had a small 3 mL vial glued to the bottom, as a place for each BA solution to be added so that GPA would not come into contact with the solution, or at least provide a physical barrier to contact with the solution. There were five replications and seven treatments: concentrations 1–5, as well as two controls, a water control and an oil control. Two late-instar/adult aphids were placed into each jar, along with one leaf, taken from *Brassica* plants free of GPA infestation, and cut in half to fit inside the 60 mL SPME jars.

Once all the aphids, BA solutions and leaves had been placed into SPME jars, they were randomized within each replicate as to their position within the growth chamber and kept at 23°C on a 16/8 (day/night) hour photoperiod. After one week in the growth chamber, one replicate was

randomly selected and tested using SPME to determine the achieved headspace concentration. After testing was complete, all samples were frozen to cause cessation of aphid biological activities. The individuals in each jar were counted and a rudimentary five-point scale assessing leaf health was developed. A rating of one meant both halves of the leaf were completely degraded or yellowed, whereas five represented both halves were still green and turgid.

BWA experiment

This test was completed in June 2015. BWA-infested logs were collected from two different Christmas tree plantations in Allegheny County, NC (36.356972, −81.232002) and brought back to Raleigh, NC. Veitch and Fraser fir branch clippings were also collected from Allegheny County, NC on the same trip. This allowed Fraser and Veitch fir treatments to be included to simulate conditions that BWA naturally develops in upon emerging from winter diapause.

Bark discs with BWA egg masses were placed into each 60 mL SPME jar, equipped with the same 3 mL vial for the 100 µL of BA solution. The same concentrations were used as in the above GPA concentration experiment. Before placing BWA-infested bark disks inside the SPME jars, each disk was inspected. Any unenclosed eggs within the egg mass were tallied, as well as any live crawlers already present on the disks. In total, there were 13 treatments. Nine of those treatments had the bark disks stuck to transparent adhesive tape, to reduce the crawlers' mobility. Concerns about the chemicals, specifically the plasticizers used in the making of the tape, led to the addition of four more treatments to determine if there is a negative effect on egg eclosion. The treatments with tape included five concentrations (the same as from the aphid experiment), a water control, an oil control, a treatment that had a defoliated Fraser fir clipping placed in the SPME jar and one that had a defoliated Veitch fir clipping added to the jar. Weights for the Fraser fir and Veitch fir clippings varied from 1.085 to 1.477 g. The treatments without tape were concentrations 1, 3, and 5, plus an oil control.

Once all treatments were added and all BWA egg information was tallied, the jars were randomized and placed into a growth chamber kept at a constant 20°C for one week, and then removed from the chamber and frozen. Each jar was then inspected, and all remaining live eggs were counted as well as any crawlers that could be found using a dissecting microscope. For treatments with tape, the tape was removed and crawlers that had adhered to the tape were counted. The bottom and sides of each jar were also inspected for crawlers.

Several changes were made to compile the data for statistical analysis. In many cases, there were more individuals present at the end of the experiment than there were at the beginning, meaning more were produced as the experiment continued. To account for this, adjustments to the number of uneclosed eggs were made. For example, if there were 18 individuals (crawlers + eggs) at the beginning of the experiment, and there were 16 crawlers and 4 uneclosed eggs at the end, the adjusted number of eggs counted in the data analysis was 2, because there were 20 individuals present at the end of the experiment, so clearly more eggs had been laid and eclosed during the experiment. Also the percent differences in uneclosed eggs from start to finish, as well as percent difference in individuals from start to finish were tabulated.

Statistical analysis

For both experiments, JMP Pro software (version 11.2.0) was used to analyze the data. For the aphid experiment, an analysis of covariance, with leaf health serving as the covariate, allowed the normalizing of the aphids present to a common mean of leaf health. The sources of variation were treatment (concentration of BA), leaf health rating (covariable, 1–5 scale) and rep. Tukey–Kramer honestly significant differences (HSD) were also used to make all pairwise comparisons between the treatments.

The BWA experiment required two layers of statistical analyses. The first was to determine whether or not there was a tape effect. A multi-way ANOVA was used to conduct this analysis with the following sources of variation: rep, treatment, tape, and all interactions. Upon determining that those treatments with tape included were not significantly different from the same treatments without tape was excluded, a complete analysis could be done. For this analysis, a multi-way ANOVA was used with rep, treatment, and all interactions as the sources of variation, along with a Tukey–Kramer HSD analysis to make all pairwise mean comparisons.

Results

GPA experiment

Raw data collected for the GPA experiment were counts of all individuals of all life stages. The analysis of variance for the entire model (p-value <.0001) was significant. Examining the F test for the sources of variation, both treatment and leaf health were significant (Table 1) at the $p < .01$ level. Least squares means, using leaf health as a covariate, and significance level for all treatment means, along with standard errors, are shown in Table 2. As BA concentration rose, individuals produced decreased, indicating a negative impact of BA on GPA reproduction. Concentration 1, control 1, and control 2 all resulted in significantly greater numbers of aphids produced than concentration 3, 4, and 5 (Table 2). Figure 2 shows a comparison of the peak signals of BA among concentrations 1, 3, and 5, tested by randomly selecting the fourth rep, and documents the differences in environmental conditions among the treatments.

Table 1. Analysis of variance of mean GPAs present with all sources of variation and associated F-ratios and p-values within the aphid experiment.

Source of variation	DF	F-ratio	Prob. > F
Treatment	6	8.33	<.0001***
Leaf health rating	1	18.37	.0003**
Rep	4	1.90	.1446

**Significant at the alpha = .01 level.
***Significant at the alpha = .0001 level.

Table 2. Pairwise comparisons of mean GPAs present (least squares mean) of all treatments and associated standard errors for the aphid experiment.

Treatment	Significance*	Mean aphids present	Standard error
Control 1 (water)	A	17.08	1.48
Control 2 (oil)	A	17.01	1.40
Concentration 1[a]	A	16.24	1.44
Concentration 2[b]	A B	12.48	1.48
Concentration 3[c]	B	9.55	1.40
Concentration 4[d]	B	8.30	1.42
Concentration 5[e]	B	8.15	1.40

*Levels not connected by the same letter are significantly different (alpha ≤ .05).
a = 100 µL of 1 µL BA in 10 mL oil solution.
b = 100 µL of 1 µL BA in 2.5 mL oil solution.
c = 100 µL of 1 µL BA in 1 mL oil solution.
d = 100 µL of 10 µL BA in 1 mL oil solution.
e = 100 µL of 25 µL BA in 1 mL oil solution.

Table 3. Analysis of variance for BWA experiment comparing treatments with tape to those without tape. Sources of variance and associated *F*-ratio and *p*-values are presented.

Source of variation	DF	*F*-ratio	Prob. > *F*
Rep	4	4.39	.0071**
Tape	1	2.02	.1664
Treatment	3	0.41	.7437
Treatment × Tape	3	0.83	.8299

**Significant at the alpha ≤ .01 level.

Table 4. Analysis of variance for mean BWA uneclosed eggs with all sources of variation and associated *F*-ratios and *p*-values presented.

Source of variation	DF	F-Ratio	Prob. > *F*
Rep	8	9.21	.2678
Treatment	4	1.41	<.0001***
Rep × Treatment	32	1.63	.1274

***Significant at the alpha ≤ .001 level.

BWA experiment

In the statistical analysis to evaluate the effect of tape, the overall ANOVA model was significant at *p*-value <.0603. Inspecting the effects and sources of variation, there was a significant rep effect, but tape and its interaction were not significant for egg eclosion (Table 3).

Because no tape effect was found, all data were pooled and another ANOVA performed excluding tape as a source of variation. Breaking it down by sources of variation, treatment was significant but rep and its interaction with treatment were not (Table 4). Least squares means of uneclosed eggs for the treatments ranged from 1.2 for concentration 2 to 12.6 for the Veitch fir control. Tukey–Kramer HSD tests showed that the Veitch fir treatment had a significantly greater mean number of uneclosed eggs compared to all other treatments (Table 5). Mean uneclosed eggs per treatment within the BA concentration treatments ranged non-uniformly from 1.2 to 4.0. The four control treatment means for uneclosed eggs ranged from 2.8 to 12.6, with Veitch fir, as previously mentioned the only significantly different mean at 12.6 (std. error = 1.14). Figure 3 shows uneclosed eggs found on one 9 mm bark disk from the fourth rep of the Veitch fir control treatment at 75× magnification. The Fraser fir control treatment had a mean uneclosed egg count of 6.2 (std. error = 1.14) and though not different than the other treatments (except Veitch fir), the Fraser fir control was the second highest uneclosed egg mean (Table 5).

Investigating percent difference in uneclosed eggs from start to finish revealed that treatment was significant, whereas the rep and rep × treatment interactions were not (Table 6). The mean percent difference among the five concentrations varied non-uniformly from −74.06% to −92.68% (Table 7). For the four control treatments, mean percent difference changed from −24.82% to −82.54% with the only significant difference being Veitch fir at −24.82% (std. error = 6.84) difference in uneclosed eggs (Table 7). The Fraser fir control mean percent difference was −62.90% (std. error = 6.84). As with mean uneclosed eggs, Fraser fir was the next lowest percent difference in uneclosed eggs, but not significantly different from all the other treatments (except the Veitch fir

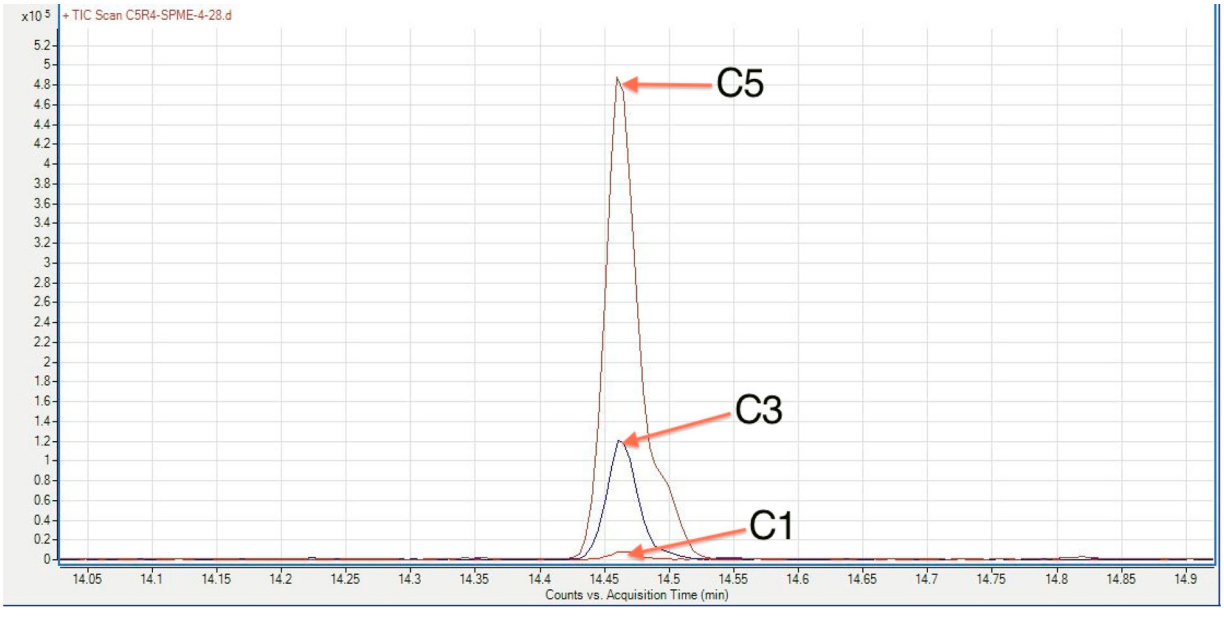

Figure 2. Comparison of BA peaks between concentration 1, 3, and 5 during the aphid experiment.

Table 5. Tukey–Kramer HSD between BWA uneclosed eggs mean (least squares mean) with standard errors reported for the BWA BA concentration experiment.

Treatment	Significance*	Mean uneclosed eggs	Standard error
Veitch fir control	A	12.6	1.15
Fraser fir control	B	6.2	1.15
Water	B	4.2	1.15
Concentration 4 [d]	B	4.0	1.15
Concentration 3 [c]	B	3.7	0.81
Concentration 1 [a]	B	3.0	0.81
Oil	B	2.8	0.81
Concentration 5 [e]	B	2.4	0.81
Concentration 2 [b]	B	1.2	1.15

Note: All concentrations and significance are according to explanations in Table 2.

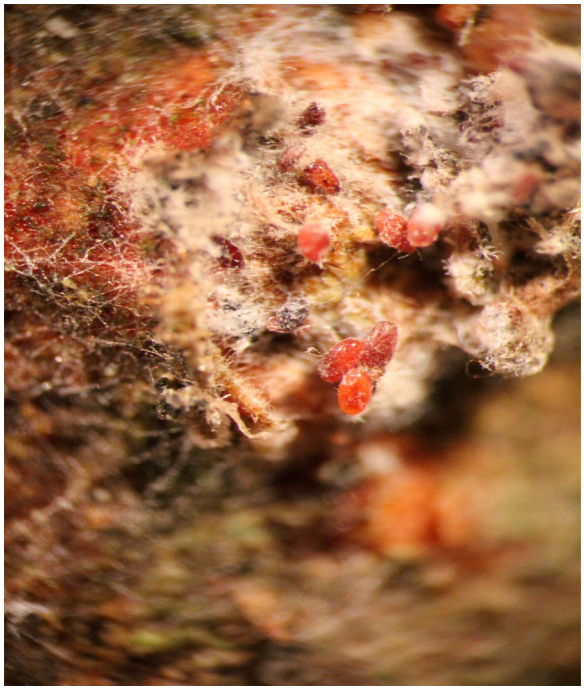

Figure 3. Uneclosed eggs on 9 mm bark disk from Veitch fir control rep 4.

control). The Tukey–Kramer HSD, and all pairwise comparisons of means of both uneclosed eggs and percent difference in eggs all indicate the only significant difference being the Veitch fir control treatment.

The second replicate was randomly chosen for SPME testing to ensure that the concentration of BA was altered. Comparing the signals shows that the concentrations among the environments were different and increased from treatment to treatment as anticipated (Figure 4).

Discussion

Aphid experiment

These results indicate a significant concentration effect of BA on the reproductive success of GPA. Altering the headspace concentration of the silicone oil solutions to roughly match the volatile signals of BA from defoliated Veitch and Fraser fir cuttings, was sufficient enough to have a significant effect on the production of GPA offspring. The approximation for the signal in Fraser fir, represented by concentration 2, was not significantly different in reduction of GPA fecundity from concentration 4, the approximation of BA's signal in Veitch fir. At the range of concentrations tested the biologic effect of BA was the reduction of fecundity rather than inhibiting reproduction altogether, a finding similar to Cates et al. (1987).

While BA did reduce fecundity, GPA is not accustomed to interacting with this particular terpenoid. Aphids are generally polyphagous feeders and studies analyzing the chemical components of the more popular food sources for GPA have not shown the presence of BA in their volatile make-up (Fernandes et al. 2009) implying that GPA has not evolved to interact with this coniferous terpenoid. It is not unknown for insects to come into contact with novel terpenoids that have a profound effect on their biology. The discovery of the "paper factor" was noted when certain insect species, after coming in contact with the paper products used within their rearing chamber were kept in a state of perpetual juvenility. This was later discovered to be related to a compound that mimics insect juvenile hormone that is present in the wood of balsam fir used to make the paper bedding lining the rearing chambers (Bowers et al. 1966). The aphid experiment yielded a useful concentration gradient, and showed that BA at concentrations naturally occurring in fir species has a negative impact on fecundity of GPA, but does not cause cessation of reproduction.

Table 6. Analysis of variance with sources of variation and associated *p*-values of significance for percent difference in eggs from start to finish during the BWA concentration experiment.

Source of variation	DF	*F*-ratio	Prob. > *F*
Rep	4	1.34	.2911
Treatment	8	9.17	<.0001***
Treatment × Rep	32	1.68	.1132

***Significant at the alpha ≤ .0001 level.

Table 7. Tukey–Kramer HSD pairwise comparisons of all mean percent differences in uneclosed eggs start to finish during the BWA concentration experiment.

Treatment	Significance*	Mean % change eggs	Standard error
Veitch fir branch	A	−24.82	6.84
Fraser fir branch	B	−62.90	6.84
Concentration 4 [d]	B	−74.06	6.84
Water	B	−76.70	6.84
Concentration 3 [c]	B	−78.06	4.84
Concentration 1 [a]	B	−82.34	4.84
Oil	B	−82.54	4.84
Concentration 5 [e]	B	−85.66	4.84
Concentration 2 [b]	B	−92.68	6.84

Note: All concentrations and significance are according to explanations in Table 2.

BWA experiment

In contrast to GPA, BWA did not show a concentration effect on fecundity associated with increasing the concentration of BA. Kumbasli and Bauce (2013) showed that while BA at higher concentrations than those found naturally in leaves reduced larval growth of spruce budworm, it alone was not enough to have toxic effects on the larval instars studied, indicating that a synergistic effect of other monoterpenes or nutrients in addition to these may be needed. In agreement with this is the Cates et al. (1987) study which saw the

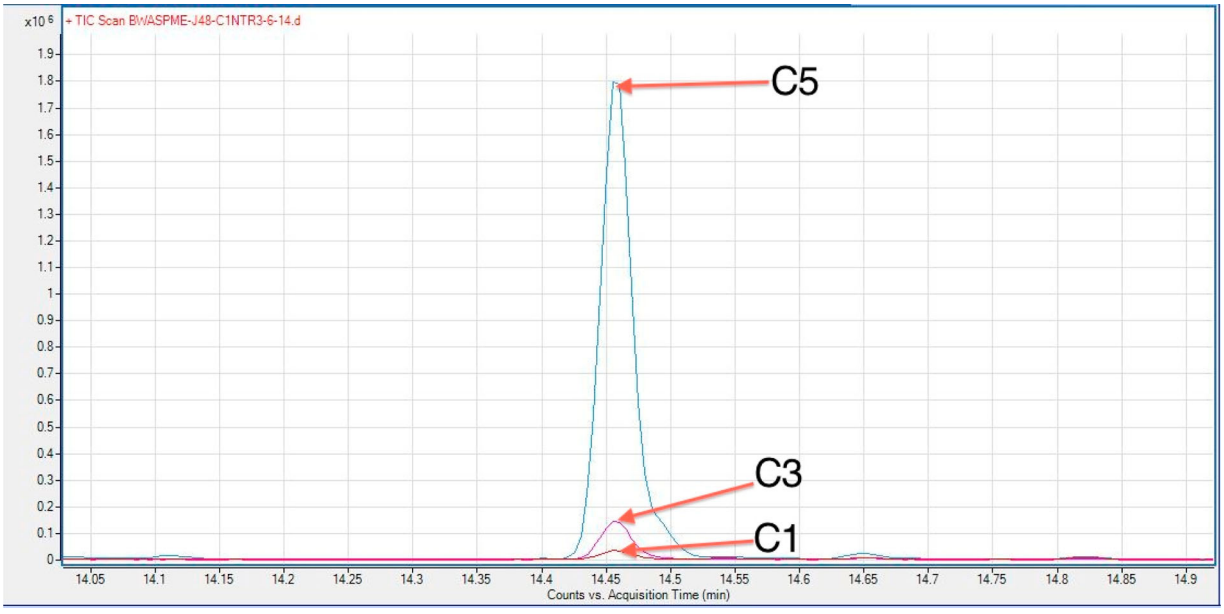

Figure 4. Comparisons between BA signal in concentrations 1, 3, and 5 during the BWA experiment.

effectiveness of BA in reducing larval growth and survival of western spruce budworm at high and low levels of nitrogen.

The effects of BA on BWA fecundity of all concentration treatments within our study were not different from the controls, outside of the Veitch fir control. This finding contrasts with results from earlier eclosion experiments (Bucholz 2015) with a known number of BWA eggs in an "open environment" (vented Petri dishes) that found that eclosion was not different between eggs placed on Veitch fir cuttings from those placed on Fraser fir cuttings. In the current experiments, the environment was sealed, which may not reflect a natural setting or open environment, but allowed the build-up of volatiles within the septum jars. Veitch fir, and, to a lesser extent, the Fraser fir treatments, indicate that the species contain volatiles, whether working singularly or in concert, that do have a negative antibiotic effect on BWA fecundity. While BA on its own does not result in a decrease in eclosion success within BWA, even at varied concentrations, the cumulative effect of many different volatiles working synergistically or perhaps a singular volatile other than BA, produced by Veitch fir, does result in significantly decreased egg eclosion success. This suggests that the volatiles alone in the headspace may have a biological effect on BWA, without the insects coming into direct contact or ingesting the compound. The Fraser fir treatment also showed a slight negative impact on BWA eclosion success, but not different from the controls. This suggests that relative abundance differences of volatiles are important to BWA resistance differences seen between Veitch and Fraser fir, but more research is needed to elucidate this finding. However, this same volatile or these volatiles do not have that kind of effect when present in an open environment (Bucholz 2015), which suggests that in nature, direct contact with or ingestion of these target volatiles may be necessary. These terpenes and other secondary metabolites generally identified through GC/MS analysis have a wide range of usefulness, from growth regulation to defense (Kačík et al. 2012). More research will be needed in order to determine the identification and importance of specific volatiles that have a negative action on the biology of BWA.

Our results also indicate that constitutive defenses may be important in resistance to BWA in fir. The cuttings used in these studies were placed on ice in a cooler for transport and kept in a cooler at temperatures just above freezing. Biological activity and production of defenses would have been limited. This suggests that those chemical defenses already prepared were enough to effect the observed change in BWA fecundity. A closer look at constitutive defenses within *Abies* could yield some target volatiles for similar analysis to ascertain biological outcomes for BWA. Constitutive defenses may be one potential resistance mechanism within the *Abies* genus against BWA.

Effectiveness of the experimental system

A novel system was developed using SPME jars to study the effects of various terpenes and terpenoids on BWA egg eclosion. Mixing terpenes with silicone GC oil enables the dilution of these terpenes for testing and creating differently concentrated environments, simulating varied levels of specific target chemicals, and allowing for the observation of changes to BWA biology. This will be a useful technique for future studies to examine singular and potentially synergistic effects of terpenes on BWA egg eclosion, as well as providing an experimental system to evaluate survival of BWA crawlers, other instars and adults in the presence of different amounts of target terpenes. It is a low cost and effective system, giving the user the ability to test headspace volatiles and create small artificial environments. This method could also potentially be useful in testing the impact of other terpenes and volatiles on a wide variety of insects, as it is an economical design and can be constructed relatively easily.

Conclusions

Concentration changes in terpene chemistry may play an important role in determining the level of resistance exhibited by a species or individual. From the GPA experiment, as the quantity of BA in the headspace was lowered, the number of offspring produced increased. As BA concentration increased in the BWA experiment, egg eclosion success was unaffected. However, Veitch and Fraser fir produce a suite of metabolites that have a negative impact on BWA egg eclosion in a closed space with Veitch fir producing a significant reduction in egg eclosion success compared to the controls. Relative concentrations and chemicals that account for the differences seen in these two species are unknown. However, the chemical differences between Veitch and Fraser fir that negatively impact BWA egg eclosion suggest that perhaps composition or relative abundance differences between these two species account for their differential impact on BWA. BA could potentially play a role, but by itself, does not appear to affect BWA egg eclosion success, at least not at the concentrations tested. Instead, our findings seem to support the notion that BA potentially works in conjunction with other components involved in the tree response chemistry to bring about any potential negative biological activity (Kumbasli & Bauce, 2013).

Pure BA has a negative effect on BWA survival (Bucholz 2015) but not at varied concentrations that approximate more natural conditions. It appears that, in our case, BA is a compound that, at varying concentrations, can reduce the number of individuals produced by GPA over a week, but alone, at varied concentrations, does not have a significant effect on egg eclosion success of BWA.

Future research projects should focus on sampling and identifying chemical defenses across a wide range of *Abies* species with known susceptibility or resistance to BWA through the use of GC/MS including SPME methodology. Comparing differences in chemical biology and relative abundance of those volatiles present will help to target specific volatiles that may be more associated with resistance. Identifying these chemicals and then examining them and their impact on BWA egg eclosion, as well as other areas of the BWA life cycle will help elucidate just what aspects of *Abies* chemical defenses are important to BWA resistance.

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