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The Influence of Dose of a Plant-Derived Volatile Cue on Arabidopsis thaliana Resistance against Insect Herbivores

Sarah Bissmeyer
University of Louisville, sebiss01@louisville.edu

Grace Freundlich
University of Louisville, grace.freundlich@louisville.edu

Christopher Frost
University of Louisville, chris.frost@louisville.edu

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Sarah Bissmeyer
University of Louisville

Grace Freundlich
University of Lousiville

Christopher Frost
University of Louisville

Abstract: *Green leaf volatiles (GLVs) are plant-derived volatile organic compounds (VOCs) known to affect plant-plant communication. Specifically, GLVs can facilitate "priming", whereby plants initiate a faster and stronger defensive response to a subsequent stress. The stress-induced GLV cis-3-hexenyl acetate (z3HAC) is a known priming cue, yet whether the concentration of the z3HAC affects plant defenses is unknown. Here, we tested the hypothesis that z3HAC concentration of would influence Arabidopsis thaliana resistance against a specialist (Trichoplusia ni) and generalist (Spodoptera exigua) herbivore. Our z3HAC treatments ranged from 0-100 ng/hr, which spanned the range of recorded natural emissions (10-40 ng/hr). We measured relative growth rates (RGR) of naïve caterpillars as a proxy for plant resistance. z3HAC concentration had no linear effects on A. thaliana resistance, even though the specialist performed better than did the generalist. Interestingly, the specialist's RGR was greater than the generalist's RGR on previously induced plants independent of z3HAC. Our results suggest that the concentration of z3HAC may not be an important factor in its reliability as a predictor of future herbivory.*

Keywords: Plant Defense, Arabidopsis, Priming, Induced Resistance

Throughout evolutionary history, plants have evolved strategies to defend themselves against herbivores and microbial pathogens (Cui et al., 2002). These strategies are essential for plant survival. However, costs associated with these strategies may inevitably lead to decreased fitness over time in the absence of their attackers (Huot et al., 2014). For instance, restricted access to resources may lead to growth-defense tradeoffs in which plants must prioritize growth or biochemical defenses, depending on environmental and metabolic conditions (Coley et al., 1985; Simms & Rausher, 1987; Herms & Mattson, 1992). Additionally, defensive compounds can be autotoxic, interfering with the growth of individuals producing them (Liu et al., 2008). A common strategy used to defer costs associated with plant defensive products is to induce them specifically

following induction by an abiotic or biotic stress against which the compounds provide resistance (Frost et al., 2008a).

One component of induced responses in plants includes the increased emission of volatile organic compounds (VOCs) (Holopainen et al. 2010). The emission of VOCs has been shown to function in alleviating oxidative plant stress (Calogirou et al., 1999; Vickers et al., 2009; Delfine et al., 2000; Loreto, 2001; Loreto & Velikova 2001), as well as serve as a direct repellant of herbivores (Beadle et al., 2006; Bleeker et al., 2009; Vancanneyt et al., 2001; Delphina et al., 2007). In addition to their role in relieving stress and directly repelling herbivores, VOCs have been validated as a reliable communication tool among plants (Baldwin et al., 1983; Frost et al., 2007). For this reason, the production of VOCs confers additional advantages to producers because these compounds may attract natural enemies of plant antagonists as well as increase relative fitness by informing other plants of the same species of an impending stress, who can then begin to accumulate defenses before the stress is experienced (Bruin et al., 2001). The phenomenon whereby a plant may begin to accumulate defenses following the reception of a reliable VOC signal prior to an induced defense response is referred to as priming (Frost et al., 2008b; Conrath et al., 2002). Priming may have fewer associated costs than the induction of a direct defense (Hulten et al., 2006). However, the adaptive significance of priming depends on the volatile cue being a reliable indicator of impending stress (Frost et al., 2008b).

One common response of plants to insect herbivory is the induced synthesis and emission of airborne VOCs, which are specifically termed Herbivore-Induced Plant Volatiles (HIPVs) (Turlings et al. 1990). HIPVs include volatile terpenes, benzenoids, and the so-called green leaf volatiles (GLVs). GLVs are 6-carbon compounds that are emitted almost immediately when herbivory occurs in almost every green leaf plant (Scala et al., 2013); GLVs form the smell of fresh mown grass. Ecologically, GLVs are an unmistakable indicator of plant damage. As such, they serve an ecological role as cues of such damage, facilitating tritrophic interactions and plant-to-plant communication (Ameye et al., 2017). In particular, the GLV cis-3-hexenyl acetate (z3HAC) has been shown to trigger defense priming (Frost et al., 2008b).

The ability to volatiles to function as stress cues is inherently related to their reliability. Reliability, in this case, signifies a predictor of future or impending herbivory. Specifically, the dose or concentration of a signal may be an important indicator of signal reliability (Girón-Calva, Molina-Torres, & Heil, 2012). The importance of dose has been recognized in medicine for centuries (Waddell, 2009): dose-dependent curves known as non-monotonic dose responses (NMDRs) have been established in organismal responses related to various hormones and endocrine disrupting chemicals (Zoeller and Vanderberg, 2015). Dose-dependency is also recognized in plant chemical ecology. For example, *Cucumbis sativus* leaves treated with increasing doses of methyl

jasmonate (MeJA) progress from traditional defense responses to mortality (Jiang et al. 2017). Moreover, volatile compounds dissipate as they move into the atmosphere, they can play discrete, dosage-specific roles over distance gradients in encounters with flower visiting animals (Galen et al., 2011). Since the responses of various metabolic systems often depend upon the dose of the chemical substances they interact with, a plant's response to a volatile cue used for priming may also be dependent upon volatile dose (Copolovici et al., 2014; Lazazzara et al., 2018; Lu et al., 2017).

It is well known that plants induce unique responses to different insect herbivore species. Specialist herbivores have been shown to outperform generalists when reared on the specialists' host plant (Kelly & Bowers, 2016; Ali & Agrawal, 2012). Therefore, the herbivore species that subsequently feeds upon the primed plant may further contribute to the defensive response elicited by plants.

Here, we use a growth chamber experiment to assess whether plant resistance is affected by dose of a known volatile priming agent. We used the model plant *Arabidopsis thaliana* with the priming volatile cis-3-hexenyl acetate (z3HAC) in response to two different herbivore species, and measured relative growth rate of herbivores as a proxy for plant defense. We hypothesized that 1) increasing dose of z3HAC would lead to a linear increase of defensive capabilities overall and 2) induced defenses to an herbivore would amplify dose-dependent resistance.

Methods

Plants and Herbivores

A. thaliana seeds were surface sterilized with 70% EtOH and then 10% bleach, and then exposed to a 48 hour vernalization period at 4°C prior to planting. Soil (Metromix 360) was autoclaved for a 90 minute cycle period. Seeds were planted in individual pots with dimensions of 5.5 x 5.5 x 5 cm3. The seeds were grown in a growth chamber with a 12:12 light:dark photoperiod and plants were monitored for a five week growth period and watered as necessary. Volatile cue doses were created from z3HAC (TCI; A0888) dissolved in lanolin (Alfa Aesar; A16902). We used eight doses: 0 (Control), 2.5, 5, 10, 20, 40, 60, and 100 ng/hr. *S. exigua caterpillars* and T. ni egg masses were obtained from Benzon Research (APHIS permit P526P-16-02563), and caterpillars were reared on artificial diet and monitored to ensure sanitary conditions during the rearing period. The oral secretion (OS) collection method was described previously (Turlings et al., 1992). Caterpillars were fed on a separate set of Arabidopsis plants for 24h prior to collecting OS. Caterpillars were held with a pair of light-weight forceps and the head region was gently pinched. The oral region of the caterpillars was held over a 0.100 mL pipet so that collections could be made. These collections

were transferred to a centrifuge tube on ice, and the OS were stored at -80°C until the experimental date. OS was diluted 1:4 with nuclease-free water prior to experimental applications (Sobhy et al., 2017; Consales et al., 2012).

Experimental Manipulations

This experiment was performed in February-March of 2017 and consisted of 48 experimental plants. *A. thaliana* plants were exposed to one of the following doses of z3HAC: 0, 2.5, 5, 10, 20, 40, 60, or 100 ng/hr. We dissolved an appropriate amount of pure z3HAC (TCA0888, TCA America) into lanolin (AAAA16902, Alfa Aesar). 50mg of this mixture was added to a 2mL glass vial with a 9mm aperture (Agilent Technologies). The concentration of z3HAC in each vial was determined to deliver the desired treatment dose in a 24h period. For the 24h volatile exposure, mature *A. thaliana* plants were moved from the growth chamber to a greenhouse where plants could be assigned to dosing chambers that were spaced to avoided overlapping treatments (Morrell & Kessler, 2017). Plants were assigned the appropriate treatment vials and were left for 24h in a dosing chamber with the vial. Each chamber consisted of a plastic box inverted to cover the plants and the vial. After 24h, vials were removed and plants were moved back into the growth chamber and randomly assigned a position in the chamber. Plants were then allowed to acclimate overnight before the oral secretion treatment. Plants within each volatile exposure group were randomly selected to receive *S. exigua* OS, T. *ni* OS, or none (control). To induce plants with OS, four leaves on each plant were first gently damaged by crushing with tweezers then 2ul of 1:4 dilution OS administered to each of 4 damage sites per leaf via calibrated pipette. After 24 hr, the four treatment leaves on each plant were carefully excised and fed to naïve caterpillars. 436 caterpillars were first weighed (pre-weight) and placed in individual containers. Individual leaves from *A. thaliana* were carefully excised from plants and placed within a container of the specified caterpillar species according to the described experimental setup. Following a 12-hour feeding period, caterpillars were collected and frozen to ensure consistency with feeding period time. Caterpillars were then weighed following this feeding period (post- weight). Relative growth rates for each caterpillar were then determined by dividing final weight from initial weight.

Statistical Analysis

Regression plots with 95% confidence intervals were determined for each experimental condition using Prism (Graphpad Software). T-tests comparing treatment pairs were conducted in both Prism and Microsoft Excel.

Results

The dose of z3HAC had an inconsistent effect on the relative growth rate for both the specialist herbivore T. *ni* and the generalist herbivore *S. exigua* (Figure 1). Specifically, we had predicted that resistance would increase with increasing dose. This was not the case. There was no relationship between relative growth rates and z3HAC dose level for either herbivore species. While there were no statistically significant effects of dose on resistance for either caterpillar species, the lowest growth for either species tended to occur in the middle ranges of z3HAC dose.

Consistent our expectation, the specialist T. ni grew better on A. thaliana than did the generalist S. exigua regardless of experimental conditions. (Figure 2). Not only did T.ni grow better than did S.exigua on A. thaliana, T.ni also had higher growth rates after application of oral secretions from either herbivore (Figure 2). That is, oral secretion pre-treatments, which were intended to induce resistance in A. thaliana, stimulated T.ni growth. In contrast, S. exigua average growth rates were slightly lower on OS-treated plants, but statistically distinguishable from non-OS controls.

Discussion

The goal of this study was to test whether a wide range of doses of a common plant-derived VOC would influence plant resistance traits against a mustard family specialist herbivore and a generalist herbivore. The results of our experiment do not provide evidence that the doses of z3HAC play a strong role in modulating resistance of Arabidopsis against herbivores.

Our hypothesis that increasing dose of z3HAC would lead to a linear increase of resistance was based on the observation that the dose of biologically relevant compounds often influences systems which presumably benefit from perceiving these compounds. For example, Cucumis sativis leaves had dose-dependent responses related to the phytohormone jasmonic acid (JA) (Jiang et al., 2017). Plants in nature can be exposed to varying concentrations of a GLV such as z3HAC, as a result of natural volatile concentration differences caused by distance gradients (Heil & Adame-Alvarez, 2010). Our specific dose selection was based on naturally wound-emitted concentrations of z3HAC cited in literature, which was 40 ng (Frost et al., 2008b). Additional reports have cited that z3HAC emissions for A. thaliana are approximately 3.72 ng/plant (Ozawa et al., 2013). For these reasons, we deliberately chose to administer doses spanning a range of biologically relevant natural dose levels. Our highest doses (100 ng/hr) even exceeds what most plants have been cited to emit. Therefore, we covered a wide concentration range from which to assess dose-dependency.

Herbivores with more specialized host ranges commonly perform better on their hosts than do generalist herbivores on those particular host plants. In our case, the more specialized herbivore, T. ni, grew better on A. thaliana than did the generalist herbivore S. exigua. This was consistent with outcomes that have been observed in literature for specialist versus generalist species. For example, past experiments have confirmed that butterfly larvae of specialists grow better and mature faster than those of generalist larvae (Hairston, 1989). That said, because we only compared two caterpillar species, we neither intended nor do we have the statistical replication to infer patterns between specialization vs. generalization in insect herbivores.

In our experiment, T. ni also performed better on A. thaliana than did S.exigua when exposed to oral secretions from either T.ni or S. exigua (Figure 2). Chemical defenses may serve as a feeding stimulant for specialist herbivores. For example, plant secondary compounds have been shown to increase feeding of specialists but not generalists (Agrawal, 2003; Mithen, Raybould, & Giamoustarius, 1995). The generalist in our study, S. exigua, performed slightly worse on average when fed on plants that had previously received S. exigua OS. Our data therefore support previous findings in literature.

An important caveat to our experiment was the method by which we fed the experimental caterpillars. In order to control feeding patterns of a large number of caterpillars (436) on a limited number of experimental plants, we directly excised from A. thaliana plants to feed to the caterpillars for RGR measurement. Removal of the leaves from the plant may have dampened the treatment effects, in part by generating a wound response in the control treatments and potentially the accumulation of secondary metabolites regulated by JA. This would be particularly true for relatively subtle ecological effects, such as volatile-mediated priming (Rowen & Kaplan, 2016; Dicke & Loon, 2000). In particular, our results provided no evidence for priming as reflected in caterpillar growth. z3HAC volatile doses spanning from 0 to 100 ng/hr were administered to individual plants, yet there was no statistical difference in the effect on priming for any of the administered z3HAC doses. This observation was counter to previous findings, which showed that z3HAC is a reliable priming cue (Frost et al., 2008b). Moreover, pre-exposure to VOCs led to significantly lower levels of growth for the caterpillar species Spodoptera littoralis (Tonn et al., 2006), showing how caterpillar growth has served as evidence for priming in former instances. So, it is possible that our experimental conditions precluded detecting some real ecological effects that might have been seen had the experiment been performed on intact plants. It is also possible that the duration of time that the volatile dose was administered could have affected our results; we opted for a 24h exposure consistent with previous literature (Frost et al., 2008b). That said, the range of doses that we used would be reasonably predicted to generate a volatile-induced resistance phenotype. In other words, the dose of z3HAC

In summary, we tested the hypothesis that dose of a GLV would directly influence plant resistance, but we were unable to support this hypothesis.   Oral secretions appeared to stimulate growth of the more specialized herbivore, while having a modest negative effect on the more generalist herbivore. There a multiple factors that may influence whether an environmental cue is a reliable indicator of a future stress. Our results suggest that dose is not an important factor for the reliability of a GLV.

Acknowledgements

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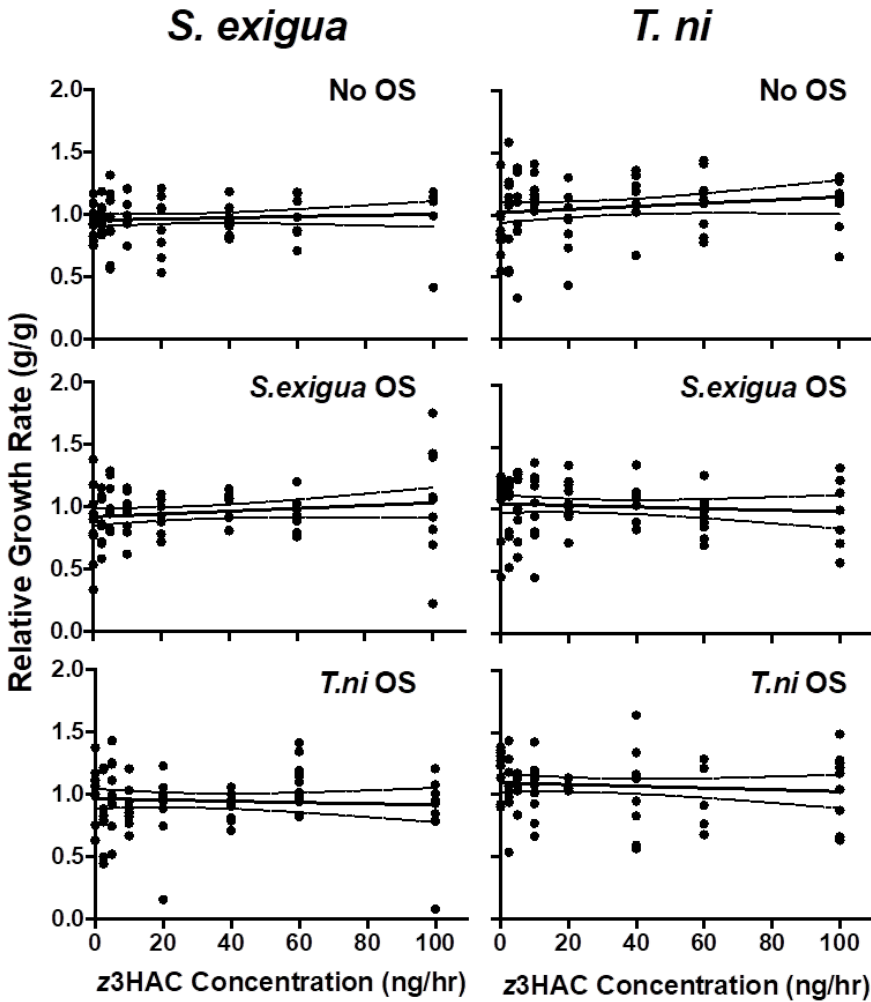
Figures

Figure 1. Relative growth rate of Spodoptera exigua and Trichoplusia ni caterpillars on Arabidopsis thaliana plants. Each plant was randomly assigned to receive a 24h dose of the green leaf volatile cis-3-hexenyl acetate (z3HAC) that ranged from 0 (control) to 100 ng/hr. After z3HAC exposure, plants were further treated with oral secretions from S.exigua (S.exigua OS) or T.ni (T.ni OS) to induce caterpillar-specific resistance phenotypes, or left as controls (No OS). Then, naïve S.exigua (left graphs) or T.ni (right graphs) caterpillars were allowed to feed for 12 hours. Relative growth rate was determined for each caterpillar, and each dot represents an individual caterpillar. Regression (solid) lines and their 95% confidence intervals (dashed lines). None of the regression line slopes significantly deviated from zero.
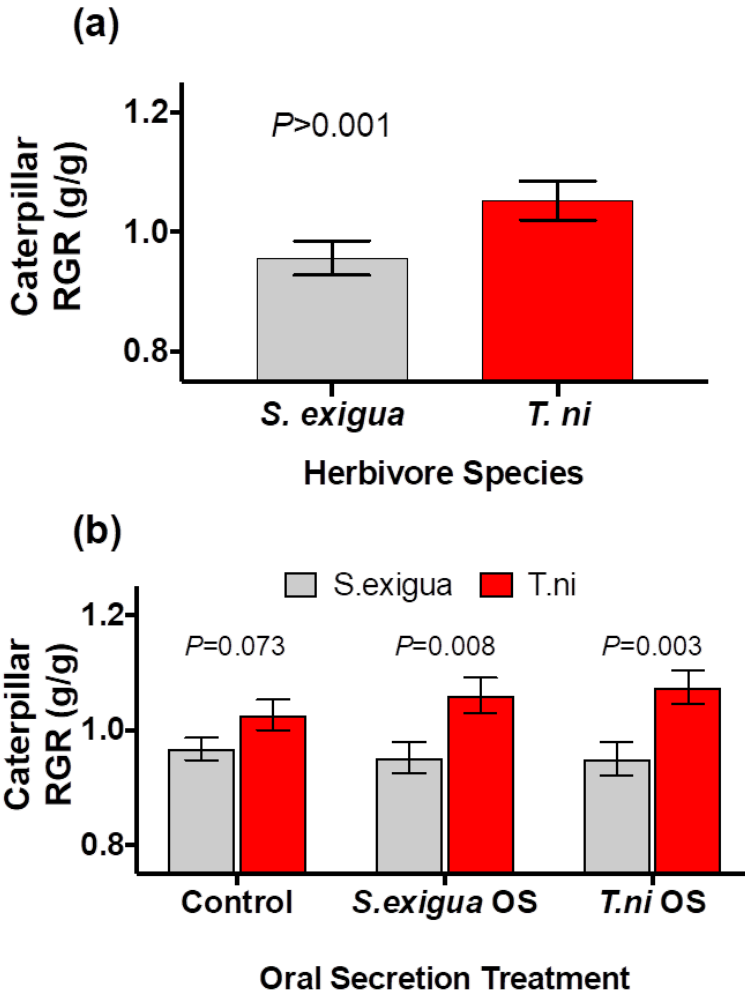
Figure 2. Relative growth rate (RGR) of a generalist (Spodoptera exigua) and a specialist (Trichoplusia ni) fed on Arabidopsis thaliana. (a) Overall RGR of all individuals of each caterpillar species. (b) RGR of caterpillars fed on A.thaliana that had been induced 24h prior with oral secretions from S.exigua or T.ni, or plants without OS (controls). Bars represent means +/- SEM. Gray bars represent S.exigua caterpillars; red bars represent T.ni caterpillars. P-values were determined by t-tests between paired groups.