Acute toxicity of the plant volatile indole depends on herbivore specialization

Running title: Insecticidal activity of plant volatile compounds

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Abstract

Herbivore-induced plant volatiles (HIPVs) provide direct benefits to plants as antimicrobials and herbivore repellents, but their potential as direct toxins to herbivores is unclear. Here we assayed the larvicidal activity of six common HIPVs from three different biochemical pathways and tested the hypothesis that the larvicidal activity of HIPVs is related to the host specialization of the insect pest. We first assessed β -caryophyllene, linalool, z-3-hexenyl acetate, z-3-hexenol, e-2-hexenol, and indole against the beet armyworm (*Spodoptera exigua*), and found that indole was 7-fold more toxic compared to the other volatiles when incorporated into diet. Then, we tested the larvicidal activity of indole against six common, destructive pest caterpillars with varying host ranges. Consistent with our hypothesis, indole toxicity varied with caterpillar host range: indole toxicity was seven-fold higher in more specialized insect species relative to generalist insect species. That said, the LC₅₀ of indole was comparable to other reported anti-herbivore agents even against the generalist caterpillars. Yet, indole in headspace had neither larvicidal nor ovicidal activity on any caterpillar species tested. These results support a key ecological precept regarding tradeoffs between host specialization and chemical detoxification, and also indicate that indole functions as a direct defense against herbivores that could be potentially useful in integrated pest management strategies.

Keywords

Caterpillars, Green leaf volatiles (GLVs), Herbivore-induced plant volatiles (HIPVs), Host Range, Indole, Pest, Toxicity, Specialist vs Generalist herbivore

Key message

- We measured the direct toxicity of six common HIPVs against the beet armyworm.
- Indole was the most toxic HIPV against the beet armyworm.
- We determined the toxicity of indole against six different pest caterpillar species.
- Toxicity of indole was associated with the host preference of the insect species.
- Indole exposure in headspace had no effect on egg hatching or caterpillar survival.
- Indole has the potential to be developed as an insecticide against crop pests.

Author Contribution Statement

CJF and AKM designed research. AKM and RCP conducted experiments. AKM and CJF analyzed data and wrote the manuscript. All authors read and approved the manuscript.

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Introduction

Plants produce a remarkable variety of volatile organic compounds (VOCs) that can affect the behavior of pollinators (Schiestl and Ayasse 2001; Schiestl et al. 1999), seed dispersers (Valenta et al. 2017), and herbivores (Agrawal 2001; Vickers et al. 2009). A subclass of VOCs is herbivore-induced plant volatiles (HIPVs), which plants release in response to herbivore attack. HIPVs are typically a blend of compounds derived from multiple biosynthetic pathways including terpenes, fatty acid derivatives, and shikimate derivatives. HIPVs confer both indirect and direct defense benefits (Hare 2011), act as priming cues that activate plant defenses and reduce herbivory (Erb et al. 2015; Frost et al. 2007; Frost et al. 2008a; Frost et al. 2008b; Heil and Bueno 2007), attract natural enemies (Birkett et al. 2003; Dicke 1986; Dicke and Sabelis 1988; Schnee et al. 2006; Turlings et al. 1995; Turlings et al. 1990; Zhu and Park 2005), and can even render herbivores susceptible to entomopathogens (Gasmi et al. 2018). HIPVs also have direct defense benefits to the plants that produce them, including protecting plants from microbial and pathogen infections (Shiojiri et al. 2006; Yi et al. 2009), deterring herbivory (Beale et al. 2006; Bernasconi et al. 1998; Heil 2004; Sandra et al. 2014) and oviposition (Kessler and Baldwin 2001; Veyrat et al. 2016; Zakir et al. 2013), and reducing caterpillar growth (von Mérey et al. 2013) and food consumption (Veyrat et al. 2016) after just HIPV exposure.

The hypothesis that HIPVs directly affect insect herbivores fecundity is not new (Pichersky and Gershenzon 2002; Unsicker et al. 2009), but the direct larvicidal or ovicidal efficacy of HIPVs on insect herbivores is poorly understood. This is due in part to the fact that the consideration of diverse phytochemicals acting as a selective pressures driving insect pest feeding strategies has largely excluded volatile constituents (Endara et al. 2017; Feeny 1976;

Howard V. C. and Bradford A. H. 2003). The vast majority of insects have evolved host range specialization, feeding on only one or a few closely related species (Forister et al. 2015), while a minority of insect herbivore species has a more generalist host range. Evolutionary theory predicts that phytochemicals that are widespread among different plant taxa will be less toxic to generalist insects compared to specialists (Howard V. C. and Bradford A. H. 2003). HIPVs tend to be common across plant taxa, and some HIPVs can be pre-synthesized, stored in specialized cells in their original or conjugate forms in various types of plant tissues (Baldwin 2010; Monson et al. 2012; Ormeño et al. 2011; Sugimoto et al. 2015; Tominaga and Dubourdieu 2000), and released when herbivory disrupts cellular storage compartments (Niinemets et al. 2013). Insect pests must therefore cope with potential toxic effects of HIPVs by either direct ingestion or headspace exposure.

The insect order Lepidoptera (butterflies and moths) contains many of the major agricultural pests that cause significant damage and economic loss of food crops worldwide (Vreysen et al. 2016). Known lepidopteran pests include both feeding specialists and generalists. To combat these pests, potent and toxic synthetic chemicals are frequently used in current agricultural systems (Cordero et al. 2006; Ecobichon 2001; Pimentel 1996). However, these insecticides can have detrimental health effects on humans and non-target organisms (Cimino et al. 2016; Hahn et al. 2015; Mulé et al. 2017; Tingle et al. 2003), and the evolution of resistance by insect pests against commonly used insecticides is well documented (Brown 1958; Sparks and Nauen 2015). Plants produce a variety of chemicals with insecticidal activity, and even some VOCs are directly toxic against invertebrates (Hubert et al. 2008; Laquale et al. 2018; Lee et al. 1999; Zhao et al. 2017). Moreover, blends of plant essential oils containing major

constituents of HIPV blends (Maffei et al. 2011) are known ovicidals and larvicidals against lepidopteran pests (Bakkali et al. 2008; El-Zaeddi et al. 2016; Isman 2016; Mossa 2016). Although the potential toxicity of individual HIPVs against lepidopteran pests is limited, investigating the larvicidal and ovicidal activity of common individual HIPVs may add to our arsenal of chemical-mediated pest control options in agriculture systems.

Here, we evaluated the hypothesis that HIPVs are acutely toxic to insect herbivores. Plant volatiles may affect herbivores either as a constituent of ingested leaf tissues or through air contact alone (Veyrat et al. 2016), so we conducted dose-response assays with HIPVs either in headspace alone or infused directly into diet. We specifically selected six HIPVs that represented the three major biochemical pathways: terpenes, green leaf volatiles (GLVs) derived from the lipoxygenase pathway, and indole (Heil 2014). Indole is an aromatic, bicyclic, amino acid precursor from the shikimate pathway. Volatile indole is emitted from a variety of eukaryotes and prokaryotes (Lee et al. 2015), and recently indole has been implicated in plant defense priming and direct defense against insects (Erb et al. 2015).

First, we tested the larvicidal activity the six individual HIPVs against a common lepidopteran herbivore pest beet-armyworm (*Spodoptera exigua*). We used the beet armyworm (*S. exigua*) in our first experiments because it is destructive generalist agricultural pest (Liburd et al. 2000; Pearson 1983) that has developed resistance against chemical insecticides (Brewer et al. 1990; Che et al. 2013), and is also a model herbivore in HIPV-mediated direct and indirect plant defense studies (Christensen et al. 2013; Engelberth et al. 2004; Huffaker et al. 2013; Jurriaan et al. 2007; Schmelz et al. 2003). As non-volatile terpenes and phenylpropanoids are known direct defenses (Moghaddam and Mehdizadeh 2017), we

predicted that indole and the terpenes would be relatively more toxic than the GLVs. Results from this experiment led us to focus our work specifically on indole. We tested the larvicidal activity of indole on six agriculturally important caterpillar species with different host ranges. Because indole is produced by a wide range of plant species (Cna'ani et al. 2018; Lee et al. 2015), we hypothesized that indole toxicity would increase with herbivore host specialization. That is, specialists would be more sensitive to indole than would be generalists. Lastly, we tested the ovicidal effect of indole. Since HIPVs provide indirect defenses by attracting egg predators (Fatouros et al. 2008), we predicted that indole would provide a direct defense benefit by reducing egg hatching success.

Materials and Methods

Plant volatiles

We used six common, commercially available HIPVs belonging to different biosynthetic pathways. Three compounds were GLVs derived from the lipoxygenase pathway: *cis*-3-hexenol (97%) (CAS: 928-96-1; TCI America), *cis*-3-hexenyl acetate (99%) (CAS: 3681-71-8; TCI America), and *trans*-2-hexenal 98%) (Sigma-Aldrich). Two terpene representatives were the sesquiterpene β -caryophyllene 97% (CAS: 87-44-5; MP Biomedicals) and the monoterpene linalool (97%) (CAS: 78-70-6; Alfa Aesar). Finally, we tested the nitrogen-containing compound indole (97%) (CAS: 120-72-9; TCI America) that derives from the shikimic acid pathway.

Experimental insects

For our experiments, we selected six common pest herbivores that are known to cause severe economic losses. We chose four generalists: beet armyworm (*Spodoptera exigua*) (Capinera 1999a; Greenberg et al. 2001), fall armyworm (*Spodoptera frugiperda*) (CABI ; Capinera 1999c), cotton bollworm (*Helicoverpa zea*) (CABI ; Martin et al. 1976), and tobacco budworm (*Heliothis virescens*) (Capinera 2001; Harding 1976; Martin et al. 1976). We selected the cabbage looper (*Trichoplusia ni*), a generalist that has a strong host preference for the mustard family (Brassicaceae) (Capinera 1999b; Hoo et al. 1984; Martin et al. 1976). Finally, we selected velvetbean caterpillar (*Anticarsia gemmatilis*), a specialist on legumes (Slansky 1993) (Waters and Barfield 1989). Eggs or egg masses of these species were obtained from Benzon Research Inc. USA (Permit #P526P-16-02563 to CJF). Eggs were immediately transferred to 2-ounce diet cups for hatching. The diet cups were maintained on shelving in a climate-controlled room at 24°C-27°C until egg hatched, and 1st instar larvae were used within 24 h of hatching for all experiments.

Preparation of test diets for feeding bioassays

Larvicidal effects of HIPVs against *S. exigua* were tested at five different concentrations 1, 2.5, 3.75, 5, and 10 mg/ml or µl/ml in feeding and headspace bioassays. Previous work establishing the LC₅₀ of *trans*-2-hexenal against five species of stored-product beetles (Hubert et al. 2008) was used as a starting point for initial test concentrations in our study, and preliminary assays suggested this range would be adequate to determine LC₅₀ for the HIPVs. However, the relatively high larvicidal activity of indole in our initial experiments caused us to also include lower concentrations of indole ranging from 0.005 to 1 mg/ml. All test diets were

prepared 12 h prior to starting an experiment. Artificial diet powder (Southland Products Incorporated, Arkansas, USA) was prepared per manufactures instructions and aliquoted into 50-ml centrifuge tubes. Prior to the diet solidifying, an appropriate amount of an individual HIPV was added, and the tube was vortexed thoroughly to mix each test compound in the diet. Control diets were prepared similarly but without any HIPV added. After solidifying at room temperature, the diet was cut into disc-shaped pieces (10 mm diameter, 5 mm height, ca. 400 mg) using a 10-cm long cork borer. Each experimental cup received one piece of artificial diet.

Preparation of volatile dispenser for headspace bioassays

Experimental amounts of *cis*-3-hexenol, *cis*-3-hexenyl acetate, β -caryophyllene, linalool, *trans*-2-hexenal, and indole was added into a 2.0ml amber glass vial (Agilent Technologies) with 1 mg of glass wool (Fig. 1). Control dispensers had only glass wool without any volatile (Erb et al. 2015). The amber vials with HIPVs were sealed with a rubber septum and connected to the diet cup by piercing the diet cup and amber vial rubber septum with an 18-gauge needle (inner diameter 0.83 mm). This design was similar to previous work in which dispensers containing 20 mg of indole were pierced with a 1 μ L micro-pipette (inner diameter 0.2mm) (Ye et al. 2019).

Test of toxicity of plant volatiles against caterpillars

First instar larvae were used for testing the toxicity of plant volatiles because the first instar is the most sensitive stage to secondary plant chemicals (Zalucki et al. 2002). For feeding bioassays, ten first instar larvae were placed in a 2 oz diet cup containing either volatile infused diet or control diet. The cup was the experimental unit of replication. Headspace

bioassays were conducted similarly, except that all diets were control (no HIPVs) and the diet cups were connected to a dispenser that contained a specific HIPV or no HIPV (control). Each experimental group had 5-10 replicate cups. The percent survival at 24 h was determined for each replicate.

Effects of headspace indole on *S. exigua* and *T. ni* egg hatching rates

For egg hatching assays, we specifically selected caterpillar species most susceptible and tolerant to indole in feeding bioassays. The inhibitory effect of indole on the beet armyworm (*S. exigua*) and the cabbage looper (*T. ni*) egg hatching was measured in a headspace bioassay. *S. exigua* and *T. ni* eggs were transferred to diet cups that were connected to volatile dispensers containing different concentrations of indole: 0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 15 and 20 mg. Each concentration of indole had 5 replicate diet cups. The percent hatch of the eggs was measured at 96 h after exposure and compared to controls without indole.

Statistical analyses

All analyses were conducted in R version 3.4.1 (R 2018) using RStudio version 1.0.153 (Wickham 2011). For calculating the median lethal concentration (LC₅₀), the mortality rates of caterpillar larvae after 24 hours of VOC exposure were regressed against indole concentration using quadratic logistic regression (glm function), and the median lethal concentration (LC₅₀) was calculated using the fitted function in quadratic logistic regression. We used this model because it produced the best fit based on AIC values. Figures were plotted with GraphPad Prism version 8.0.0 (San Diego, California USA) and ggplot2 in R Studio (Wickham 2011).

Results

HIPV toxicity against *S. exigua*

Indole caused highest larval mortality of all six HIPVs tested (Fig. 2A). With the exception of β -caryophyllene, all HIPVs that were directly consumed in diet caused complete mortality at some concentration tested (Fig. 2A). In contrast, no HIPV showed any toxicity to *S. exigua* when administered in headspace alone at any concentration (Fig. 2B). Based on LC_{50} values, indole was more than 7 times more larvicidal than was the second most potent volatile (linalool) among all the HIPVs tested against *S. exigua* (indole LC_{50} = 0.35 mg/ml; linalool LC_{50} = 2.59 μ l/ml) (Fig. 3). GLVs were relatively less toxic: *cis*-3-hexenol (LC_{50} = 3.32 μ l/ml diet), *cis*-3-hexenyl acetate (LC_{50} = 4.61 μ l/ml diet) and *cis*-3-hexenal (LC_{50} = 4.85 μ l/ml diet) (Fig. 3). β -caryophyllene was neither toxic in diet nor headspace against *S. exigua* caterpillars at any of the tested concentrations. Mortality of *S. exigua* was negligible in the control group.

Indole toxicity relative to caterpillar host range

The caterpillar species with restricted host ranges were more sensitive to indole than were the generalist caterpillars. The LC_{50} of indole for the four widely generalist pests ranged from 0.18-0.35 mg/ml diet (Fig 4). Specifically, the LC_{50} of indole was 0.35 mg/ml for *S. exigua* (Fig. 4A), 0.29 mg/ml for *S. frugiperda* (Fig. 4B), 0.27 mg/ml for *H. zea*. (Fig. 4C), and 0.18 mg/ml for *H. virescens* (Fig. 4D). In contrast, the LC_{50} of indole was 0.05 mg/ml for both the velvetbean caterpillar (*A. gemmatilis*) and the cabbage looper (*T. ni*) (Fig. 4E,F). That is, indole was 3.6-7.0 times more toxic for the two more specialized caterpillars than it was for the four generalists.

Larvicidal and ovicidal activity of volatile indole in headspace

Consistent with our first experiment using *S.exigua* (Fig. 2B), indole present only in headspace had no effect on *T. ni* caterpillar mortality (Fig.5). Furthermore, there was no inhibitory effect of any tested concentration of indole on egg hatching success of either *S. exigua* or *T. ni* caterpillars (Fig. 6A,B). Because these two caterpillar species were the most (*S.exigua*) and least (*T.ni*) sensitive to indole, we did not conduct headspace bioassays for mortality or egg hatching on the other caterpillars.

Discussion

We have demonstrated that (1) HIPVs have direct larvicidal activity in an HIPV-specific manner, (2) the larvicidal effect of HIPVs is largely constrained to direct consumption by caterpillars as opposed to volatile exposure alone, and (3) the larvicidal effect of the common volatile indole depends on the degree of host specialization of the caterpillar species. To our knowledge, this is the first study to report LC₅₀ values for HIPVs against lepidopteran pests, which was a key goal of this study. In supporting our prediction that larvicidal activity would vary among HIPVs, we found that indole and, to a lesser extent, GLVs and volatile terpenes, had direct larvicidal activity against the beet armyworm, a destructive pest with a wide host range. The larvicidal effect of some GLVs and terpenes has been reported against stored-pest beetles (Hubert et al. 2008) and aphids (Sadeghi et al. 2009), and our results are comparable. For example, the LC₅₀ values we obtained for the three GLVs tested, *cis*-3-hexenol (3.32 µl/ml), *cis*-3-hexenyl acetate (4.61 µl/ml), and *trans*-2-hexenal (4.85 µl/ml) (Figure 3), are

similar to those reported against stored pest beetles (0.6-3.32 mg/g) (Hubert et al. 2008). Similarly, the larvicidal activity of the terpene linalool against *S.exigua* in our study (LC₅₀ of 2.59 µl/ml) is comparable to previous work testing linalool against the European corn borer (Lee et al. 1999). This suggests that the LC₅₀ values we obtained may be broadly applicable across insect taxa.

A key finding is that indole is considerably more toxic than the other HIPVs tested. In fact, the LC₅₀ of indole (50-350 µg/ml) is comparable to other natural toxicants such various strains of *Bacillus thuringiensis* (LC₅₀ = 63.0-153.0 µg/ml) (Moar et al. 1989) and purified Cry1 protein from *B.thuringiensis* (LC₅₀=1-870 µg/g) (Ali et al. 2006; Niu et al. 2013). Moreover, the LC₅₀ of commercial *B. thuringiensis* DiPel ES (LC₅₀= 2 µg/g)(Liao et al. 2002) and the synthetic insecticide lambda-cyhalothrin (LC₅₀=5.27 µg/ml)(Hardke et al. 2011) are close to LC₅₀ obtained for indole at 24h in our experiments. While the LC₅₀ values of tested HIPVs in this study are higher than emission rates observed in nature (Allmann et al. 2013; Degen et al. 2012), they are likely representative of what might be stored within leaf tissues (Loreto et al. 1998; Loreto et al. 2000; Niinemets et al. 2004) and what insect herbivores may realistically encounter in their natural diets. In other words, our results have both ecological and practical relevance.

In our study, indole in headspace alone did not affect caterpillar survival or egg hatching. In fact, none of the volatiles tested showed any effect when present in headspace alone (Fig. 2B). In previous work, volatile indole reduced survival of the generalist herbivore *S. littoralis* by ~10% in neonates and ~6% in 1st instar (Veyrat et al. 2016). This study also noted decreases in food consumption and, surprisingly, increases in larval weigh under indole

treatments (Veyrat et al. 2016). While we did not find similar effects with *S.exigua*, our study was focused on larvicidal activity as reflected in LC_{50} , which is fundamentally different metric. So, it appears that volatile exposure may exert non-lethal influences on caterpillar fecundity. That said, exposure of eggs to indole in headspace also had no effect on hatching success of either *S.exigua* or *T.ni*. (Figure 6), which is consistent with previous work (Veyrat et al. 2016). Plants volatiles can clearly have repellent effects on insect pests (Beale et al. 2006; Bernasconi et al. 1998; Heil 2004; Sandra et al. 2014), but our study indicates that larvicidal efficacy of HIPVs depends on their direct consumption by herbivores.

The larvicidal activity of indole varied with caterpillar host range in a manner that supported our initial prediction. A long-standing hypothesis is that generalist herbivores are well-equipped to detoxify wide array of common phytochemicals (Agrawal and Ali 2012; Krieger et al. 1971), whereas specialist herbivores are more tolerant to compounds specific to their host range but sensitive to more common phytochemicals (Whittaker and Feeny 1971). In other words, there is a tradeoff between chemical detoxification and host specialization. Since free indole is produced in some eukaryotes and prokaryotes (Lee et al. 2015) and is a common HIPV (Cna'ani et al. 2018; Frost et al. 2007), we predicted that it would be relatively toxic to specialist caterpillars compared to generalist caterpillars. Indeed, larvicidal activity of indole was approximately seven times lower for specialist *A. gemmatalis* compared to generalist *S. exigua* and, in general, all the generalist species were tolerant to indole relative to the specialist. The exception was *T. ni*, a pest that displays clear feeding preferences for Brassicaceae species (Rivera-Vega et al. 2017), which had an LC_{50} to indole approximately same as the specialist *A. gemmatalis* (LC_{50} =0.05 mg/ml of diet) (Figure 4). While we only used

six caterpillar species in our study, our results support ecological theory related to herbivore specialization.

Indole, and other HIPVs, may be useful insecticides in agricultural systems. More than 500 insect pest species have developed documented resistance to chemical insecticides (Georghiou 1990). Agriculturally destructive caterpillars are particularly capable of developing such resistance (Ahmad et al. 2008; Che et al. 2013; Elzen 1997; Hardee et al. 2001; McEwen and Splittstoesser 1970; Yu et al. 2003). Plant secondary metabolites are among the alternatives to synthetic insecticides in pest management, while also potentially avoiding or ameliorating negative impacts on beneficial organisms. Our study identifies that indole, and potentially other plant-derived volatiles, may be important additions to the arsenal of chemical defenses in pest management. Due to its toxicity, indole might be particularly useful as part of integrated pest management strategy for both generalist and specialist caterpillars given its larvicidal effect is similar or stronger than to some commercial pesticides and potent biopesticides like the Cry1F *bacillus thuringiensis* protein. Even the larvicidal activity of the GLVs and linalool against *S.exigua* in our study is approximately the same as reported for other pests. Therefore, our results indicate that HIPVs warrant attention as chemical components of control strategies against insect pests.

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Figure legends

Fig. 1 Volatile delivery system for headspace bioassay.

Fig. 2 Direct toxicity of plant volatiles at concentrations on the survival of *S. exigua* in feeding bioassays (A) and headspace bioassay (B). Values at each concentration represent the mean of five-ten biological replicates $\pm$ 1SEM.

Fig. 3 LC₅₀ of individual plant volatiles on *S. exigua* caterpillars in feeding bioassay. Graphs for each volatile refer to fitted values based on quadratic logistic regression. LC50 represent lethal concentration causing 50 percent mortality. Data are reproduced individually from Fig.2A for easy visualization.

Fig. 4 Direct toxicity of indole on the survival of five different caterpillar species in feeding bioassays. Values at each concentration represent the mean of five-ten biological replicates $\pm$ 1SEM. *S. exigua* data are reproduced (dashed lines) from Fig. 2 to aid in comparison.

Fig. 5 Effect of varying concentrations of indole on the survival of *T. ni* in headspace bioassays. Values at each concentration represent the mean of five-ten biological replicates $\pm$ 1SEM.

Fig.6 Effect of varying concentrations of indole on percent egg hatch of *S.exigua* (A) and *T. ni* (B). Values represent mean of five biological replicate $\pm$ 1SEM.

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