

# Determining Defense Priming of Squash Plants (Cucurbitaceae: Cucurbitales) Against Salt Marsh Moth (Lepidoptera: Erebidae)

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**Abstract:** In response to being fed on by herbivores, plants produce volatile compounds that play important roles in plant defense. A relatively recent discovery revealed that undamaged neighboring plants can detect these volatiles as a warning of possible future herbivore attack and respond by priming their anti-herbivory defenses. Primed plants typically mount a faster or stronger defense after an herbivore begins feeding. Plant volatile-mediated priming has been observed in many plant species but has not yet been documented in the family Cucurbitaceae. Therefore, we sought to test if squash plants (*Cucurbita pepo*) respond to volatiles from herbivore-damaged plants by priming their defenses. Our objective was to evaluate plant priming in response to herbivory. For our objective, emitter plants were either damaged by specialist salt marsh moth caterpillars (*Estigmene acrea*) or left as undamaged controls. Neighboring “receiver” plants were exposed to damage or control emitter volatiles for 24 hours and then challenged with herbivory by larvae. We quantified the amount of herbivory on receivers and found a difference in damage between volatile exposed receivers and unexposed receivers. As well as a significant presence of salicylic acid in the exposed receivers before infection. Taken together, our findings contribute to a better understanding of plant defense priming and offer insight into how plants predict and defend against different herbivore species

*Keywords: Herbivory, Estigmene acrea, plant defenses, priming*

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Although they are immobile creatures, plants have multiple means of defense against herbivory. Most defenses are commonly thought to be constitutive, or always present on a plant. However, plants have become highly adapted to respond to cues created by their environment as well as aggressors (Mescher & Pearse 2016). Inducible defenses are triggered by these cues or by direct attack from assailants (Mithöfer & Boland 2012). In recent years, priming has been shown to induce plant defenses in response to these

signals from pathogen attack or herbivory. Little is known about the specifics of how these cues are used by plants to evoke defense mechanisms, yet they are essential for plants to prepare for potential herbivore attacks. Priming is the physiological process by which a plant prepares to respond to future biotic or abiotic stress more efficiently (Frost et al. 2008). Volatile organic compounds (VOCs) are released by emitter plants once herbivory, movement, or egg-laying is detected. These VOCs are dependent on the

emitter plant as they are either produced naturally or in direct result of herbivory. Those VOCs produced due to herbivory are called herbivore induced plant volatiles (HIPVs). HIPVs can play a large role in multitrophic interactions, as they not only elicit responses from carnivores, parasitoids, and pollinators, but as they communicate the possibility of harm to neighboring plants (Dicke & Baldwin 2010). Specific HIPVs have been identified recently in crop such as maize, in which the aromatic compound Indole is responsible for the induction of the defense compound jasmonic acid among neighbor plants (Erb et al. 2015). Other means of defense can be induced such as defense gene expression (Arimura et al. 2000, Godard et al. 2008), increased production of VOCs to attract predators (Bruin et al. 1992, Glinwood et al. 2009, Birkett et al. 2000), decreased attractiveness (Glinwood et al. 2004, 2009), increase in phenolic compounds (Baldwin & Schultz 1983), and a general natural resistance to herbivory (Rhoades 1983, Dolch & Tschardtke 2004, Karban et al. 2000, 2004). The overall goal for this research was to determine whether or not the zucchini plant (*Cucurbita pepo* var. *Raven F1*) responds to HIPVs by priming of their defenses. More so it was hypothesized that receivers exposed to damaged-emitter plants would sustain less feeding damage relative to neighboring plant exposed to undamaged-emitter plants. This research sought to better understand the roles of VOCs, specifically HIPVs, in cucurbit defenses against herbivores.

## Materials & Methods

Salt marsh moth caterpillars were utilized in this experiment based on their diverse diet of crop plants. Neighbor 'receiver' plants were exposed to the volatiles produced by either larva damaged or undamaged emitter plants

for 24 hours. Those receiver plants were then infected with their own larva for 24 hours. Tissue samples as well as damaged leaves were taken to determine the specific defense mechanisms used by zucchini plants.

## Plants & Insects

Salt marsh moth larvae used in the experiment were the offspring of a parental generation collected from a plot of land in Hillsboro, TX. Larvae were reared on zucchini squash plants prior to experimentation and kept in mesh caging until reaching the correct instar stage. Plants used in the experiments were zucchini squash of the Raven F1 variety (Johnny Selected Seeds, Fairfield, ME) approximately three weeks old. Zucchini squash plants were raised in a greenhouse setting with a light schedule consisting of 18 hours of day and 6 hours of night.

## Volatile Exposure

Three plants, two receivers and one emitter, were equally spaced in a 99.37 L Plastic bin (Sterilite, Townsend, MA). Emitter plants were placed into two groups, either treatment or control. Treatment emitters were damaged by four 5<sup>th</sup> instar salt marsh moth larvae feeding for 24 hours, control emitter plants were left undamaged for the same amount of time. Larvae were bagged onto leaves using 4 x 4.72 Organza bags (Amazon, Amazon.com) to prevent the movement from emitter to receiver plants. Control plants were bagged as well. After the first 24 hours, all emitter plants were removed from their bins, tissue samples (~ 100 mg) from one leaf of the receiver plants were collected and placed into liquid nitrogen for later processing. Each receiver was then fed on by four 3<sup>rd</sup> instar larvae, two bagged on two separate leaves and left for 24 hours. Once the 24 hours ended, larvae were removed from receiver plants and another tissue sample was collected from one of the eaten leaves where

damage was present. The second damaged leaf was cut from the base of the stem and taped onto a piece of printer paper (Office Depot, Boca Raton, FL) for damage analysis. These leaves were stored in a -40 °C freezer for preservation and to prevent the leaf from browning. There were five replicates each for treatment and control groups.

### Leaf Area Damage Analysis

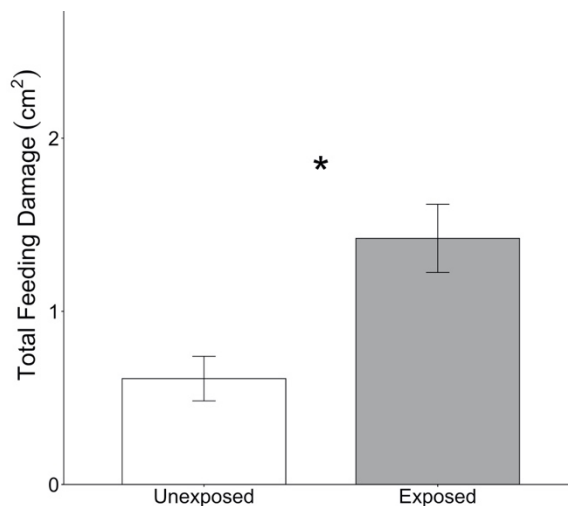
Damaged leaves recovered from receiver plants were analyzed to calculate the amount of damage sustained from the salt marsh moth larvae. Damage was colored in black and traced in white to be recognized by the ImageJ computer program (National Institutes of Health, New York, NY). Once uploaded onto ImageJ, the area of each damaged spot on the leaves was measured and placed into an Excel spreadsheet. The total amount of damage and average amount of damage per bin was calculated. Both the average and total amount of damage was used to assess the

### Quantification of Phytohormones

The tissue samples collected from the receiver plants both prior to and after feeding damage were used to quantify the amounts of Jasmonic acid (JA) and Salicylic acid (SA) present in the plant. Samples were placed into liquid nitrogen after collection and stored in a -80 °C freezer to preserve the chemical state of the leaf. Extraction of JA and SA as well as the quantification of the two organic compounds was replicated as previously described in Schmelz et al. 2003, 2004. Plant hormones were extracted to methyl esters, which were isolated and extracted in a vapor phase. The compounds were analyzed by a GC/CI-MS (Agilent Technologies, Santa Clara, CA), quantifying the amounts of JA and SA for each sample. The presence of these compounds was confirmed when comparing the retention times of the samples with standards of the compounds.

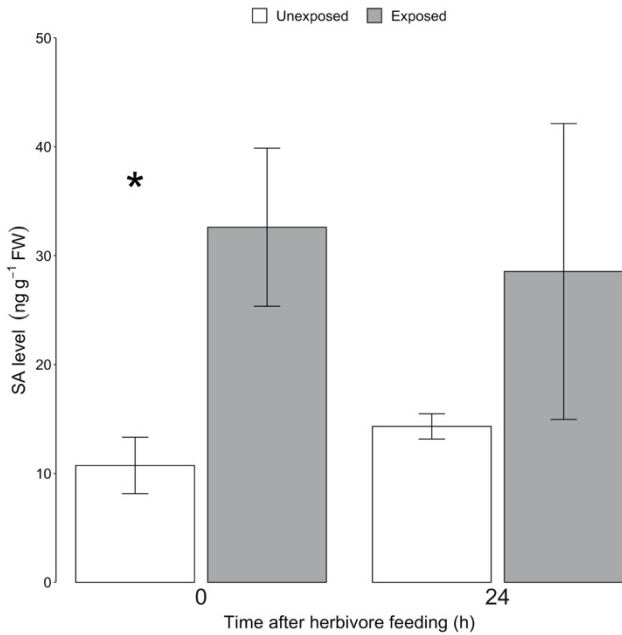
## Results

Once the priming trials were completed and leaf tissue was collected for phytohormone samples, the second damaged leaf was assessed for total area damage from each receiver plant. Both control and treatment receivers were compared in the amount of feeding damage sustained from salt marsh moth feeding to determine the effects of volatile exposure. It was found that treatment receivers, exposed to emitter plants damaged by salt marsh moth larvae, endured significantly more damage ( $P = .0396$ ) than their unexposed counterparts. The amount of damage in control receivers was almost half the amount of damage in treatment receivers (Figure 1). With the addition to the leaf area



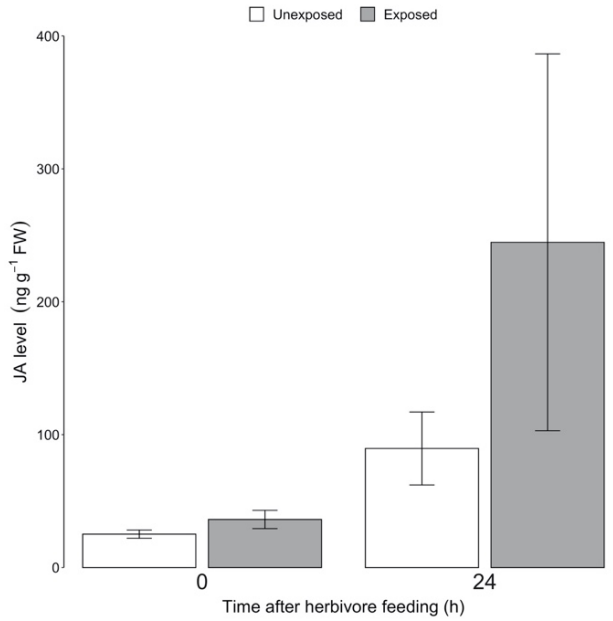
**Figure 1** Exposure to HIPVs significantly increases salt marsh larvae feeding damage on neighboring plants. (n=20; n=10 unexposed control plants across 5 bins; n=10 exposed treatment plants across 5 bins)

damage analysis, phytohormone samples were collected using leaf tissue from before and after salt marsh moth larvae fed on receiver plants. Leaf tissue was collected to assess the presence of two important plant defense hormones, JA and SA. Once processed and ran through GC/CI-MS, the samples revealed that there was a significant



**Figure 2** Exposure to HIPVs induces increases in SA (n=19; n=10 unexposed control plants across 5 bins; n=9 exposed treatment plants across 5 bins) but does not prime changes in SA following larvae feeding (n=20; n=10 unexposed control plants across 5 bins; n=10 exposed treatment plants across 5 bins).

amount of SA ( $P = .00682$ ) before feeding in exposed receiver plants compared to unexposed receivers. The amount was no longer significant after 24 hours but is still larger than exposed receivers. In Figure 2 you can see that the amount of SA increases in the unexposed receivers and decreases in the exposed receivers. As for JA, there was no significant difference in amount of the hormone in either the exposed receivers or unexposed receivers before feeding damage. JA levels were higher in the exposed receivers ( $P = .188$ ) compared to unexposed receivers ( $P = 0.389$ ). Over the next 24 hours, the amount of JA increased in both the



exposed and unexposed plants (Figure 3). The JA levels in exposed receivers were higher but not significant

## Discussion

Our results demonstrate that there is significantly more feeding damage inflicted onto receiver plants that have been exposed to salt marsh moth larvae infected emitter plants. Furthermore, it was found that receiver plants contain a significant amount of SA prior to herbivore feeding. HIPVs have the ability to prime a plants defense through different defense mechanisms. The JA pathway within plants can be induced due to mechanical damage, hence HIPVs communicate potential injury to neighboring plants (Ruan et al. 2019). However, an induction of the SA pathway signifies that the zucchini plants are preparing for pathogen attack rather than herbivores (An & Mou 2011). Plant volatiles are typically thought to act as positive regulators in a plant's defense system through priming or inducing a plant's defenses (Erb 2018). Yet, the results suggest that neighboring plant's being attacked could be sabotaging their neighbors or their own defense systems by communicating a mistaken threat. Priming using neighboring

VOCs has even been considered to be eavesdropping on the receiver's part (Dicke & Baldwin 2010).

These results also refute our original hypothesis, that exposure to these herbivore induced plant volatiles will prime the defenses of the neighboring squash plants. Priming is specifically characterized to occur after attack, or as soon as feeding occurs (Frost et al. 2008). Although the amount of SA is significantly large before subjecting the receivers to larvae, the amount decreases the following 24 hours. This occurrence rather explains defensive induction, when a chemical compound is induced once a plant is harmed (Arimura et al. 2005). Figure 3 demonstrates there is a insignificant increase in JA from 0 to 24 hours, which can

comparatively suggest squash plants are priming their defenses against the larvae.

SA and JA are commonly used to identify the existence in priming, yet other chemical compounds and even volatile organic compounds (VOCs) can express priming. VOCs play an important role in both indirect defenses, such as recruiting natural enemies, and indirect defenses, in our case of priming (Arimura et al. 2005). It is possible there is a different defense pathway within cucurbits that are demonstrating priming. Specialized plant metabolites, such as Cucurbitacin C, which is known to create a bitter taste upon consumption (Shang et al. 2014). More experiments are being conducted to characterize the specific VOCs associated in these plant-herbivore interactions.

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