The Piercing-Sucking Herbivores *Lygus hesperus* and *Nezara viridula* Induce Volatile Emissions in Plants

Livy Williams III,* Cesar Rodriguez-Saona, Paul W. Paré, and Steven J. Crafts-Brandner

Plant volatiles induced by herbivory are often used as olfactory cues by foraging herbivores and their natural enemies, and thus have potential for control of agricultural pests. Compared to chewing insects and mites, little is known about plant volatile production following herbivory by insects with piercing-sucking mouthparts. Here, we studied factors (insect life stage, gender, the role of salivary glands, and type of bioassay used for volatile induction) that influence the induction of plant volatiles by two agriculturally important hemipterans, *Lygus hesperus* and *Nezara viridula*. Feeding on intact cotton by virgin females of *L. hesperus* induced 2.6-fold greater volatile response compared to that induced by mated females, possibly due to increased feeding activity by virgin females. This plant volatile response was associated with elicitors present in the insect’s salivary glands as well as to the degree of mechanical injury. Feeding injury by *N. viridula* females also increased volatile emissions in intact maize by approximately 2-fold compared to control plants. Maize seedlings injured by *N. viridula* emitted higher amounts of the monoterpene linalool, the sesquiterpenes (E)-β-caryophyllene, α-trans-bergamotene, and (E,E)-β-farnesene, and the homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, but not amounts of green leaf volatiles, compared to uninjured plants. Emissions from intact maize injured by adult males were lower than those emitted by adult females of the same age and did not differ from those emitted by uninjured plants. Similarly, feeding by virgin female *N. viridula* followed by excision led to 64% higher quantities of volatiles compared to untreated plants. Volatile emission in excised plants, however, was considerably greater than in intact plants, suggesting that careful consideration must be given to bioassay design in studies of herbivore-induced plant volatiles. Salivary gland extracts of *N. viridula* led to sesquiterpene emissions approximately 2.5-fold higher than for controls, although no significant differences were observed for green leaf volatiles, monoterpenes, and homoterpenes. These results indicate that *L. hesperus* and female *N. viridula* feeding induce volatile production in plants, and that volatile production is affected by gender and life stage of the bug. Although oviposition and mechanical injury by stylets may increase release of volatiles, elicitors from salivary glands of *L. hesperus* and *N. viridula* also seem to play a role in the emission of plant volatiles.


**Keywords:** *Lygus hesperus*, *Nezara viridula*, Miridae; Pentatomidae; plant volatiles; herbivory; salivary glands; volicitin

INTRODUCTION

Plants are often exposed to an array of potential enemies, including pathogens, arthropods, and vertebrates. One mechanism of plant defense involves the production of volatiles that may repel herbivores and/or attract the natural enemies of the herbivores (Turlings et al., 1995; DeMoraes et al., 1998; Kessler and Baldwin, 2001). Injury by phytophagous insects and spider mites increases the emission of volatiles in plants (Paré and Tumlinson, 1997a,b; Dicke, 1999; Hilker and Meiners, 2002). This response occurs at the site of injury as well as systemically (i.e., from uninjured portions of injured plants), and may persist for several days (Loughrin et al., 1994; Paré and Tumlin-
son, 1998; Schmelz et al., 2001). The release of herbivore-induced volatiles appears to involve the de novo synthesis of compounds that are not emitted from uninjured or mechanically injured plants (Röse et al., 1996; Paré and Tumlinson, 1997b). Several biosynthetic pathways are activated by herbivory, and these often involve the isoprenoid pathway (terpenes), the shikimic pathway, and the lipoxygenase pathway (green leaf volatiles). In addition, the response of plants to different species of herbivores may be specific. Plants may respond differently to different instars of the same herbivore species (e.g., Takabayashi et al., 1995) or may respond differently to herbivory by closely related species of herbivores (e.g., DeMoraes et al., 1998).

Most of our knowledge to date on herbivore-induced volatiles has come from studies of mites and chewing insects, especially lepidopterous larvae and, more recently, phloem-feeders (Homoptera) (Table 1). However, the volatile response of plants to other insects with piercing-sucking mouthparts (Hemiptera) has received relatively little attention. Feeding by Lygus hesperus (Hemiptera: Miridae) has been shown to induce local and systemic emission of a blend of plant volatiles (Rodriguez-Saona et al., 2002; Blackmer et al., 2004) that was similar to that produced by caterpillar feeding (Paré and Tumlinson, 1997a,b). Treatment of excised plants with L. hesperus salivary gland extracts (systemic uptake via the transpiration stream) induced the same volatile blend as plants exposed to feeding by bugs or treated with volicitin, an elicitor in caterpillar oral secretions (Alborn et al., 2000). Plants exposed to reproduc-

### Table 1. Species of Herbivorous Arthropods in Which Chemical Analysis of Induced Plant Volatile Emissions Has Been Documented*

<table>
<thead>
<tr>
<th>Species Common name</th>
<th>Order</th>
<th>Type feeding</th>
<th>Host plant</th>
<th>Induced volatiles?</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Panonychus ulmi</td>
<td>Acari</td>
<td>Cell content feeder</td>
<td>Apple</td>
<td>Yes</td>
<td>Llusia and Peñuelas (2001)</td>
</tr>
<tr>
<td>Tetranychus urticae</td>
<td>Acari</td>
<td>Cell content feeder</td>
<td>Various</td>
<td>Yes</td>
<td>Dicke et al. (1990)</td>
</tr>
<tr>
<td>Leptinotarsa decemlineata</td>
<td>Coleoptera</td>
<td>Chewing</td>
<td>Potato</td>
<td>Yes</td>
<td>Bolter et al. (1997)</td>
</tr>
<tr>
<td>Popillia japonica</td>
<td>Coleoptera</td>
<td>Chewing</td>
<td>Grape</td>
<td>Yes</td>
<td>Loughrin et al. (1996)</td>
</tr>
<tr>
<td>Ostrinia nubilalis</td>
<td>Coleoptera</td>
<td>Chewing</td>
<td>Maize</td>
<td>Yes</td>
<td>Turlings et al. (1998)</td>
</tr>
<tr>
<td>Epitrix hirtipennis</td>
<td>Coleoptera</td>
<td>Chewing</td>
<td>Tobacco</td>
<td>Yes</td>
<td>Kessler and Baldwin (2001)</td>
</tr>
<tr>
<td>Liriomyza trifoli</td>
<td>Diptera</td>
<td>Chewing</td>
<td>Bean</td>
<td>Yes</td>
<td>Finidori-Logi et al. (1996)</td>
</tr>
<tr>
<td>Lygus hesperus</td>
<td>Hemiptera</td>
<td>Piercing-sucking</td>
<td>Various</td>
<td>Yes</td>
<td>Rodriguez-Saona et al. (2002)</td>
</tr>
<tr>
<td>Dioryctys mimimus</td>
<td>Hemiptera</td>
<td>Piercing-sucking</td>
<td>Tobacco</td>
<td>Yes</td>
<td>Kessler and Baldwin (2001)</td>
</tr>
<tr>
<td>Nezara viridula</td>
<td>Hemiptera</td>
<td>Piercing-sucking</td>
<td>Bean</td>
<td>Yes</td>
<td>Colazza et al. (2004b)</td>
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<tr>
<td>Rhopalosiphum padi</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Wheat</td>
<td>Yes</td>
<td>Quiroz et al. (1997)</td>
</tr>
<tr>
<td>Cacopsylla</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Various</td>
<td>Yes</td>
<td>Scutareanu et al. (2003)</td>
</tr>
<tr>
<td>Psylla</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Pearl</td>
<td>Yes</td>
<td>Scutareanu et al. (1997)</td>
</tr>
<tr>
<td>Rhopalosiphum maidis</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Maize</td>
<td>Yes</td>
<td>Bernasconi et al. (1998)</td>
</tr>
<tr>
<td>Rhopalosiphum maidis</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Maize</td>
<td>No</td>
<td>Turlings et al. (1998)</td>
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<tr>
<td>Asylophron phorpus</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Broad bean</td>
<td>Yes</td>
<td>Du et al. (1998)</td>
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<tr>
<td>Trialeurodes vaporariorum</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Bean</td>
<td>Yes</td>
<td>Birkett et al. (2003)</td>
</tr>
<tr>
<td>Bemisia tabaci</td>
<td>Homoptera</td>
<td>Sucking</td>
<td>Cotton</td>
<td>No</td>
<td>Rodriguez-Saona et al. (2003)</td>
</tr>
<tr>
<td>Hoplocampa testudinews</td>
<td>Hymenoptera</td>
<td>Chewing</td>
<td>Apple</td>
<td>Yes</td>
<td>Bove et al. (1996)</td>
</tr>
<tr>
<td>Cydia pomonella</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Apple</td>
<td>Yes</td>
<td>Herr and Dorn (2001)</td>
</tr>
<tr>
<td>Heliothis virescens</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Various</td>
<td>Yes</td>
<td>De Moraes et al. (1998)</td>
</tr>
<tr>
<td>Helicoverpa zea</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Various</td>
<td>Yes</td>
<td>De Moraes et al. (1998)</td>
</tr>
<tr>
<td>Spodoptera littoralis</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Maize</td>
<td>Yes</td>
<td>Turlings et al. (1998)</td>
</tr>
<tr>
<td>Spodoptera exempta</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Various</td>
<td>Yes</td>
<td>McCall et al. (1994)</td>
</tr>
<tr>
<td>Pieris rapae</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Various</td>
<td>Yes</td>
<td>Shojiiri et al. (2001)</td>
</tr>
<tr>
<td>Plutella xylostella</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Cabbage</td>
<td>Yes</td>
<td>Shojiiri et al. (2001)</td>
</tr>
<tr>
<td>Mythimna separata</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Various</td>
<td>Yes</td>
<td>Fujimura et al. (2000)</td>
</tr>
<tr>
<td>Manduca sexta</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Tobacco</td>
<td>Yes</td>
<td>Takabayashi et al. (1995)</td>
</tr>
<tr>
<td>Pieris brassicae</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Various</td>
<td>Yes</td>
<td>Mattiacci et al. (1994)</td>
</tr>
<tr>
<td>Pseudoletia separata</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Corn</td>
<td>Yes</td>
<td>De Moraes et al. (1998)</td>
</tr>
<tr>
<td>Pieris napi</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Nasturtium</td>
<td>Yes</td>
<td>Gavrel et al. (1997)</td>
</tr>
<tr>
<td>Manduca quinquemaculata</td>
<td>Lepidoptera</td>
<td>Chewing</td>
<td>Tobacco</td>
<td>Yes</td>
<td>Kessler and Baldwin (2001)</td>
</tr>
</tbody>
</table>

*For each herbivore species, the type of feeding habit, host plant from which volatiles were collected, whether plant volatiles were induced by arthropod feeding, and a cited reference are presented.

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tively mature female bugs produced a volatile blend with a greater composition of constitutive compounds (e.g., α-pinene) than plants exposed to nymphs or adult males, suggesting that injury to plant tissue during oviposition also contributed to volatile production (Rodriguez-Saona et al., 2002). Thus, it appears that the life stages of bugs, gender, and perhaps reproductive status of female *L. hesperus* affect plant volatile production and release.

Olfactometer trials demonstrated that a *Lygus* egg parasitoid, *Anaphes iole* (Girault), spent more time in odor fields of *L. hesperus*-injured plants than untreated controls (Manrique et al., 2005). Colazza et al. (2004a,b) demonstrated that feeding by the stink bug *Nezara viridula* (Hemiptera: Pentatomidae) induced a volatile blend similar to that described for *L. hesperus*. Feeding by *N. viridula* induced significantly more volatile production in *Vicia fabae* than in *Phaseolus vulgaris*. For both plant species, a combination of feeding and oviposition increased emission of (E)-β-caryophyllene over plants with feeding injury alone. However, the effect of the reproductive status of *N. viridula* females on plant volatile induction is not known.

Specificity in the activation of volatiles in plants might be closely associated with the herbivore’s feeding habit. For instance, when comparing three herbivores with different feeding habits, Turlings et al. (1998) found that phloem feeders (aphids) caused no increase in volatile emissions from maize, whereas injury by a species of caterpillar and a stemborer increased volatile emissions. Similarly, we have collected volatiles from cotton plants injured by three species of herbivores with different feeding habits: a chewing caterpillar *Spodoptera exigua*, a piercing-sucking hemipteran *Lygus hesperus*, and the phloem feeder whitefly *Bemisia tabaci* (Rodriguez-Saona et al., 2002, 2003). The phloem feeder did not elevate volatile emissions from cotton. In contrast, injury by the chewing caterpillar and the piercing-sucking hemipteran increased volatile emissions from plants (Rodriguez-Saona et al., 2002, 2003; Fig. 1).

Bioassay design can also have dramatic effects

**Fig. 1.** Headspace volatile emissions from cotton plants injured by three species of herbivores with different feeding habits: the chewing caterpillar *Spodoptera exigua*, the piercing-sucking bug *Lygus hesperus*, and the phloem feeder *Bemisia tabaci*. Volatile compounds are: 1 = (E)-2-hexanal, 2 = α-pinene, 3 = β-pinene, 4 = myrcene, 5 = (Z)-3-hexenyl acetate, 6 = limonene, 7 = (E)-β-ocimene, 8 = linalool, 9 = (E)-4,8-dimethyl-1,3,7-nonatriene, 10 = indole, 11 = (E)-β-caryophyllene, 12 = α-humulene, 13 = (E)-β-farnesene, 14 = (E,E)-α-farnesene, 15 = nerolidol, 16 = (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Bars represent the mean ± 1 SE. Data extracted from Rodriguez-Saona et al. (2002, 2003).
on plant volatile production and release. Schmelz et al. (2001) found that excised maize leaves produced 2.5- to 8-fold higher volatile response than did intact leaves. It is apparent that bioassay design has important implications for both basic and applied investigations, and thus deserves further study to better understand its role in studies of induced plant responses.

We conducted a study using gas chromatography-mass spectroscopy (GC-MS) to expand our understanding of plant volatile production in response to feeding by two agriculturally important hemipterans: *L. hesperus* and *N. viridula*. We addressed the following specific questions: (1) Do plants respond differently to virgin and mated *L. hesperus* females? (2) Is mechanical injury sufficient to induce plant volatiles or are elicitors in *L. hesperus* salivary glands necessary? (3) Does *N. viridula* feeding induce volatiles from intact and excised plants? (4) Is plant response to *N. viridula* injury affected by life stage and gender? (5) Do *N. viridula* salivary gland secretions induce volatile production in plants?

**MATERIALS AND METHODS**

**Plants**

Cotton (*Gossypium hirsutum* L. var. Delta Pine-land 5415) was grown in greenhouses for 4–5 weeks until plants had 4–5 fully expanded leaves, as described in Rodriguez-Saona et al. (2002). Maize (*Zea mays* L. var. LG-11) seedlings, 12–14 days old, were grown in a plant growth room in 16-cm diameter pots with Pro-Gro potting soil. Plants were fertilized with 8 g Osmocot 14-14-14 fertilizer (Scotts-Sierra Horticulture, Marysville, OH) at the time of planting. Light (700 μmol photons/m²/sec) was provided by high-pressure sodium and metal halide lamps. The temperature in the growth room was maintained at 29 ± 4°C, 40 ±10% RH, and 16:8 L:D photoperiod.

**Insects**

*Lygus hesperus* Knight was collected from alfalfa fields (Maricopa, AZ) and maintained in the laboratory under the conditions described by Rodriguez-Saona et al. (2002). *Nezara viridula* (L.) was obtained from a laboratory colony begun from field collections in Washington, Bolivar, and Sunflower counties, Mississippi. Bugs were swept from soybean (*Glycine max* (L.) Merr.), cotton, and non-crop hosts from May through October. Prior to 1 November, bugs were maintained at 25 ± 1°C, 65–95% RH, and 16:8 L:D photoperiod. To simulate winter conditions, bugs were held at 10 ± 1°C, 65–95% RH, and 8:16 L:D photoperiod from 1 November to 15 February. After 15 February, bugs were returned to the 25 ± 1°C, 65–95% RH, and 16:8 L:D photoperiod regime. Voucher specimens of *L. hesperus* and *N. viridula* are deposited in the National Entomological Collection, National Museum of Natural History, Smithsonian Institution, Washington, DC.

In the laboratory, *N. viridula* was reared in cages constructed from 19-l white plastic buckets, tree seedling protector tubes (Ben Meadows Co., Janesville, WI; product no. 4JB-152470), and fiberglass window screen. The seedling protector tubes were cut to 32-cm lengths and seven tubes (32 × 8 cm) were placed in a bucket. One 30 × 30 cm paper towel (Kimwipes EX-L, Kimberly-Clark Corp., Roswell, GA; product no. 34133) was folded and placed in each tube. The seedling protector tubes and paper towels increased surface area inside the cage and provided an oviposition substrate for the bugs. A 50-cm-square piece of window screen was placed on top of the bucket and held in place with a drawstring. A mesh plastic bag containing approximately 40 g of raw shelled peanuts (*Arachis hypogaea* L.) was placed on the center of the screen and fresh green bean (*Phaseolus vulgaris* L.) pods were placed on the screen around the bag. Placing the food on top of the screen allowed the bugs to feed through the screen, while avoiding accumulation of decaying food inside the cage. This setup also facilitated replenishment of the food without opening the cage. Bean pods were replaced daily and peanuts were replaced weekly. Bugs were transferred into clean cages weekly. About 50 bugs were reared in each cage.
Collection and Analysis of Volatiles From Intact Plants

Previously, we showed that injury to cotton by female *L. hesperus* induces emissions of volatiles (Rodriguez-Saona et al., 2002). In the present study, cotton volatile emissions were compared when injured by virgin adult females (<4 days old) and mated females (>4 days old). Thirty to 40 *L. hesperus* females were placed on plants 18 h prior to volatile collections. Volatiles were collected, as described in Rodriguez-Saona et al. (2002), the following day commencing at 1000 h. Volatiles from *L. hesperus*-infested cotton were collected continuously for 8 h and analyzed as described in Rodriguez-Saona et al. (2002). Each treatment was replicated 5–7 times.

Volatiles were collected from maize seedlings injured by five *N. viridula*. Maize was used because previous studies showed induction of volatiles from maize injured by *L. hesperus* (Rodriguez-Saona et al., 2002) and use of maize facilitated direct comparison of treatment effects between *N. viridula* and *L. hesperus*. Volatile emissions were collected from three treatments: plants with no insect injury (controls), plants injured by virgin females, and plants injured by virgin males. Virgin adults were removed daily from cages of maturing 5th instar nymphs and held individually in 500-ml transparent plastic food containers (Solo Cup Co., Urbana, IL) with ventilated lids, and were provided with bean pods. Adult age of the bugs used in this study ranged from 2–25 days with an average of 14 days. Virgin adults were used because results with *L. hesperus* indicated that virgin females induce higher emissions of volatiles compared to mated individuals (see below), and also because our goal was to collect only those volatiles induced by insect feeding, and not by oviposition. Insects were allowed to feed on maize plants for 24 h prior to experiments to become conditioned to their new diet. Insects were then transferred to uninjured plants and allowed to feed for 24 h (0900 to 0900 h) before volatile collection was begun (see below). This work was conducted in the same plant growth room described above. Insects were removed from plants prior to placing plants in collection chambers. Treatments were replicated 3–4 times.

Volatile collection was initiated at 1000 h and continued for 6 h. Volatiles were collected indoors (but not in plant growth rooms) in rooms maintained at 29 ± 4°C, 40 ±10% RH, and 16:8 L:D period with a light intensity of 700 µmol photons/m²/sec provided by high-pressure sodium and metal halide lamps. Volatiles were collected using a push-pull system (Heath and Manukian, 1994). Purified air entered the top of a 60-cm-high × 14-cm-diameter glass cylinder and passed over the plants inside the cylinders at 3 liters/min. Volatiles emitted from plants were collected by pulling air through the chambers and then through Super-Q adsorbent (Alltech, Deerfield, IL) filter traps at 1 liter/min. The remaining air was vented out through an opening at the bottom of the chambers, loosely sealed with cotton around the stem of the plant. The system allowed for simultaneous collection of volatiles from four independent plants.

After collections, volatiles were extracted from traps with 150 µl methylene chloride. Five microliters of an internal standard (n-nonyl acetate 40 ng/µl) was added. The extract (1 µl) was injected onto a 15-m × 0.25-mm ID fused silica column with a 0.1-µm-thick bonded methyl silicone stationary phase in a Hewlett-Packard 6890 gas chromatograph. Injections were made in a splitless mode for 0.3 min with an injector temperature of 230°C and a detector temperature of 250°C. The column was held at 40°C for 0.5 min, increased 12°C/min to 180°C, and then increased at 40°C/min to 220°C and held for 2 min. Helium was used as carrier gas at a linear flow velocity of 35 cm/sec. Volatiles from each sample were quantified using GC-FID. Quantification was based on comparison of their peak areas with that of the internal standard. Compounds were identified by GC-MS as described in Rodriguez-Saona et al. (2001). Spectral data were compared with synthetic standards from commercial sources and from spectra by the National Institute of Standards and Technology (NIST, 1995) database.
Collection and Analysis of Volatiles From Excised Seedlings

We conducted experiments to determine if volatiles from excised maize seedlings were comparable with those from intact plants. Maize plants were injured by *N. viridula* as described above for intact plants. Four treatments were compared: plants with no insect injury (controls), plants injured by five virgin females, plants injured by five virgin males, and plants injured by five 5th-instar nymphs. After exposure to insects, but prior to volatile collection, seedlings were excised close to the soil at the base of the stem with a sharp razor, and wrapped with wet cotton at the site of excision. This procedure constituted "post-treatment" excision. Seedlings were placed in 37-cm-long × 4-cm-diameter closed glass cylinders. Air passed over each plant inside the cylinders at a rate of 600 ml/min and was pulled through a filter trap placed at the opposite end of the chamber at the same rate. Volatiles from 8–12 seedlings were collected simultaneously in parallel glass chambers indoors under the conditions described above. Volatiles were collected for 6 h (1000–1400 h) and analyzed as described previously. Each treatment was replicated 3–4 times.

Dissection of Salivary Glands

Salivary glands of *L. hesperus* and *N. viridula* were dissected as described in Rodriguez-Saona et al. (2002). Groups of salivary glands (20 glands for *N. viridula*; 100 glands for *L. hesperus*) were each placed in 1.5-ml microcentrifuge tubes with distilled water (20 µl for *N. viridula*; 50 µl for *L. hesperus*) and held at −80°C until experimentation. Although the size of *N. viridula* salivary glands was not measured, glands of female bugs appeared to be larger than those of males, as was the case for *L. hesperus* (Rodriguez-Saona et al., 2002).

Induction of Volatiles by Salivary Gland Extracts

We used excised seedlings to test if extracts of *N. viridula* salivary glands induce volatile emissions in maize similar to those induced by *L. hesperus* using the same technique (systemic uptake by excised plants) (Rodriguez-Saona et al. 2002). Approximately 100 *N. viridula* salivary glands (mixed sexes) were placed in a 1.5-ml microcentrifuge tube with 300 µl of distilled water. The tube was sonicated for 15 min and then centrifuged for 5 min at 16,000g. After centrifugation, the supernatant was transferred to a new tube and held at −20°C until needed. Maize seedlings were excised at the base of the stem as described previously. Seedlings were incubated in 1-ml glass shell vials containing either 30 µl of stink bug salivary gland extract and 220 µl of 50 mM sodium phosphate buffer, pH 8.0, or 30 µl of distilled water and 220 µl of buffer solution (controls). Plants were incubated for 12 h at room temperature in a darkened cabinet starting at 2100 h. The following morning, seedlings were removed from vials; the cut ends were wrapped in wet cotton and then placed in 37-cm-long × 4-cm-diameter glass cylinders. Volatile collections and analyses were conducted as described previously for insect-injured excised seedlings. Each treatment was replicated 3–4 times.

We used excised seedlings to determine if application of *L. hesperus* salivary extracts to intact plants induced volatile emission. Previous incubation experiments using systemic uptake of salivary extracts by excised plants showed that salivary glands of *L. hesperus* induce volatiles from maize seedlings (Rodriguez-Saona et al., 2002). An experiment was conducted to further determine if application of salivary gland extracts on intact maize plants (a more realistic treatment) induced volatiles and if the volatile response was due to mechanical injury caused by insect feeding. Mechanical injury was mimicked by creating holes similar to those caused by *L. hesperus* injury to plants using laboratory picks (Greer Laboratories, Inc., Lenoir, NC). Plants (*N* = 8) either received mechanical injury plus salivary gland extracts or mechanical injury plus distilled water. Plants were treated the day prior to volatile collection (2100 h). For those plants that received the salivary gland extracts, picks were immersed into the extract; otherwise picks were immersed into distilled water.
Five distinct applications (one or two per leaf) were made on each seedling by gently squeezing the picks through the surface of leaves, which allowed the extract to enter the plant tissue. Each applied site received approximately 1 µl of extract or distilled water (total of 5 µl per plant). Plants were allowed to respond to the treatment overnight. The following morning (1000 h), plants were excised with a razor and volatiles were collected consecutively for 6 h and analyzed as described previously.

**Statistical Analyses**

Differences among treatments were analyzed by completely randomized one-way analysis of variance (ANOVA) (Systat ver. 9 1998; SPSS Science, Chicago, IL). Differences between two treatments were analyzed using two-tailed t-tests. Data were log-transformed prior to analysis to satisfy assumptions in ANOVA.

**RESULTS AND DISCUSSION**

Injury to cotton by virgin females of *L. hesperus* induced 2.6 times more volatiles compared to those induced by mated females (total amounts [ng/h ± SE]: virgin females = 1271.86 ± 404.50; mated females = 488.86 ± 186.37; F = 4.54; df = 1,10; P = 0.059) (Fig. 2). These results, and those reported previously (Rodriguez-Saona et al., 2002), indicated that virgin females of other piercing-sucking species caused a higher induction of plant volatiles compared to immatures or mated adults.

Feeding injury to intact maize seedlings by *N. viridula* adult females increased total volatile emissions by approximately 2-fold compared to untreated control plants (total amounts [ng/h ± SE]: control = 10.69 ± 1.45; *N. viridula* females = 21.20 ± 3.68; F = 7.14; df = 1,4; P = 0.05) (Fig. 3). Injury to intact plants induced higher amounts of the monoterpene linalool, the sesquiterpenes (E)-β-caryophyllene, α-trans-bergamotene, and (E,E)-β-farnesene, and the homoterpene (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Fig. 3A). Emissions from plants fed on by adult males were lower than those emitted by adult females of the same age and did not differ from those emitted by untreated control plants (total amounts [ng/h ± SE]: 14.46 ± 4.23; P > 0.05). Colazza et al. (2004b) also reported significant increases of (E)-β-caryophyllene and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene in two legumes, *V. fabae* and *P. vulgaris*, fed on by adult *N. viridula*. In the present study, overall concentrations of maize volatiles were considerably higher than for either of these legumes. The observed differences in volatile production between the two studies may also have been caused by differences in methods, i.e., infestation level of female bugs, or periodicity and duration of volatile collections. In other studies, maize injured by chewing insects (caterpillars and beetles) and a piercing-sucking hemipteran (*L. hesperus*) produced these compounds in higher quantities than reported in the present study (Turlings et al., 1990, 1998; Rodriguez-Saona et al., 2002). Quantitative and qualitative differences in volatile production appear to exist between different plant species injured by herbivores, even when injured by insects with similar feeding habits such as insects with piercing-sucking mouthparts. These differences may be due in part to interspecific variability in feeding behavior (Turlings et al., 1998) and oral secretions (Pohnert et al., 1999; Alborn et al., 2003; Mori et al., 2003). Taken as a whole, it appears that the plant biosynthetic pathways involved in volatile production operate differently for different plant species and herbivores.

Because excised seedlings respond to treatments differently than intact plants (Schmelz et al., 2001), plant volatiles released after stink bug feeding were compared using excised and intact plants. We found a similar qualitative response of excised seedlings (post-treatment excision) to injury by *N. viridula* compared to intact plants (Fig. 3B). However, feeding by *N. viridula* on intact plants followed by excision led to 64% higher quantities of volatiles compared to untreated controls (total amounts [ng/h ± SE]: control = 45.81 ± 5.50; *N. viridula* females = 75.04 ± 8.57; F = 8.23; df = 1,6; P = 0.028). As was observed for intact plants, *Nezara*-injured excised seedlings emitted higher amounts.

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Fig. 2. Headspace volatiles collected from intact cotton plants injured by either virgin or mated females of *Lygus hesperus*. Volatile compounds are same as described in Figure 1. Bars represent the mean + 1 SE (*n* = 5–7). An asterisk above compound number indicates a significant difference in volatile emission between treatments (*P* < 0.05); otherwise volatile emissions between treatments were not significantly different.

Fig. 3. Headspace volatiles collected from untreated maize seedlings (controls) or from seedlings injured by virgin females of *Nezara viridula*. Bioassays were conducted with intact (A) and excised (post-treatment) (B) plants. Volatile compounds are: 1 = (*E*)-2-hexanal, 2 = (*Z*)-3-hexenol, 3 = α-pinene, 4 = β-pinene, 5 = myrcene, 6 = (*Z*)-3-hexenyl acetate, 7 = limonene, 8 = (*E*)-β-ocimene, 9 = linalool, 10 = (*E*)-4,8-dimethyl-1,3,7-nonatriene, 11 = indole, 12 = (*E*)-β-caryophyllene, 13 = α-trans-bergamotene, 14 = α-humulene, 15 = (*E*)-β-farnesene, 16 = (*E*,*E*)-α-farnesene, 17 = nerolidol, 18 = (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Bars represent the mean + 1 SE (*n* = 3–4). Asterisks above compound numbers indicate significant differences in volatile emissions between control and treated plants (*P* < 0.05; **P** < 0.01); otherwise volatile emissions between treatments were not significantly different.
of the monoterpene linalool and the sesquiterpenes (E)-β-caryophyllene, α-trans-bergamotene, and (E,E)-β-farnesene compared to untreated control plants. In addition, higher emissions of (E)-4,8-dimethyl-1,3,7-nonatriene, (E,E)-α-farnesene, and nerolidol were detected from Nezara-injured excised seedlings compared to untreated control plants (Fig. 3B). No significant increases in volatil e emissions were detected when plants were injured by either N. viridula nymphs or adult males compared to controls (total amounts [ng/h ± SE]: nymphs = 61.05 ± 10.38; males = 117.09 ± 69.99; P > 0.05; data not shown). Although feeding injury for the treatments was not measured, it is possible that differential feeding between treatments led to the observed results. Our observations of increased overall production of volatile sesquiterpenes ((E)-β-caryophyllene, α-trans-bergamotene, and (E,E)-β-farnesene) in excised versus intact plants was consistent with increases in these compounds by plants treated with volicitin (Schmelz et al., 2001).

Sesquiterpene emissions from maize seedlings were approximately 2.5-fold higher after treatment with N. viridula salivary gland extracts than with controls (t = 2.59; df = 5; P = 0.048; Fig. 4). The major sesquiterpenes induced included (E)-β-caryophyllene, α-trans-bergamotene, and (E,E)-β-farnesene. Significant differences were not observed for green leaf volatiles, monoterpenes, and homoterpenes (P > 0.05, Fig. 4). This increased response in sesquiterpene emission after treatment with salivary gland extract was similar to that observed with insect feeding on intact and excised seedlings, with the exception that insect feeding also increased release of the monoterpene linalool and the homoterpenes (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and (E)-4,8-dimethyl-1,3,7-nonatriene compared to uninjured plants (Fig. 3). Schmelz et al. (2001) and Rodriguez-Saona et al. (2002) also reported increased production of the sesquiterpenes (E)-β-caryophyllene, α-trans-bergamotene, and (E,E)-β-farnesene from excised maize leaves after treatment with volicitin, a component of S. exigua oral secretions hypothesized to initiate the release of induced plant volatiles. These results suggest that mechanical injury by stylets during N. viridula feeding may result in the production and release of volatiles not induced by salivary gland extracts, such as monoterpenes and homoterpenes. In fact, emission levels of volatiles were lower in plants treated only with salivary gland extracts, as compared to plants fed on by live bugs, which also suggests that mechanical injury by stylets is an important factor in volatile production and emission.

Our previous studies showed that salivary gland extracts from L. hesperus induce volatile emissions from maize (Rodriguez-Saona et al. 2002) and cotton seedlings (unpublished data). In the present study, mechanical injury to maize alone was not sufficient to induce the response induced by L. hesperus salivary gland extracts. Mechanically-injured intact maize plants that were treated with salivary gland extracts of L. hesperus induced 2.7-fold higher emis-
sions of volatiles compared to those plants that received distilled water (controls) (total amounts [ng/ h ± SE]: mechanical injury + salivary gland extracts = 374.48 ± 67.78; mechanical injury + distilled water = 138.08 ± 26.01; t = 3.26; df = 6; P = 0.017). Our studies with L. hesperus and N. viridula salivary gland extracts suggest that mechanical injury by stylets and chemical constituents in the saliva may work in concert to initiate the production and release of induced volatiles in plants.

In conclusion, piercing-sucking hemipterans and chewing caterpillars appear to induce a similar volatile response in plants (Rodriguez-Saona et al., 2002; Kessler and Baldwin, 2004) that differs from phloem feeders (i.e., aphids and whiteflies) (Rodriguez-Saona et al., 2003) (Fig. 1). Two classes of compounds identified from caterpillar regurgitant induce volatile emissions in plants: β-glucosidases (Mattiacci et al., 1995) and several fatty acid-amino acid conjugates, including volicitin (Alborn et al., 1997; Halitschke et al., 2001; Mori et al., 2003). The elicitor(s) in N. viridula and L. hesperus salivary glands that are responsible for triggering the induction and release of volatiles have yet to be determined. Previously, we showed that the volatile blend induced by L. hesperus salivary glands is similar to that induced by volicitin (Rodriguez-Saona et al., 2002). Although our chemical analyses of the salivary glands from Lygus species and stink bugs have so far shown no evidence of volicitin being present (unpublished data), it is possible that other fatty acid-amino acid compounds are present. Overall, these studies suggest that the mechanisms of volatile induction caused by L. hesperus and N. viridula feeding are similar to that induced by chewing caterpillars, and that the volatile plant response is also similar to that induced by volicitin.

In addition, we showed that volatiles induced by piercing-sucking hemipterans vary depending on the life stage and gender, most likely due to variations in the size and constituents of their salivary glands, as well as to the amount of injury they inflict during feeding and/or oviposition. Thus, volatiles induced by piercing-sucking hemipterans seems to involve a combination of mechanical injury, due to feeding and/or oviposition, as well as elicitor(s) from salivary glands, and perhaps from female reproductive organs (e.g., Meiners and Hilker, 2000; Wegener et al., 2001). The relative importance of each of these factors on plant volatile emissions, and consequently on the foraging of herbivores and their natural enemies, remains unknown. However, recent studies have shown that Lygus-induced plant volatiles provide information to foraging conspecifics (Blackmer et al., 2004), and also play a role in host habitat location by their natural enemies (Manrique et al., 2005).

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LITERATURE CITED


