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Artificial Substrate Bioassay for Testing Oviposition of Southern Green Stink Bug Conditioned by Soybean Plant Chemical Extracts

ANTÔNIO R. PANIZZI,[1, 2] MARK BERHOW,[3] AND ROBERT J. BARTELT[4]

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ABSTRACT A laboratory bioassay was developed for testing oviposition preference of southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae), toward chemicals extracted from soybean, *Glycine max* (L.) Merrill, pods and leaves. In this bioassay, an artificial substrate (cheesecloth) was stretched over a wooden ring (embroidery hoops), treated with plant extracts or chromatographic fractions, and then exposed to adult stink bugs to assess oviposition preference. The methanol extract of pods stimulated the greatest oviposition. After a chromatographic separation on a reverse phase open column, the most active fraction derived from this extract was that eluted with 20% methanol in water. After subjecting this fraction to chromatography on silica, the greatest activity occurred in the fraction eluted with 60% methanol in methylene chloride. Further fractionation of this material by thin layer chromatography gave no single fraction with demonstrated activity, but the recombined fractions were again active, indicating that multiple components are probably involved in eliciting oviposition. Antennectomized females did not differentiate treated versus untreated substrates, but females with the hairs of the genitalia coated did, indicating that the oviposition-eliciting compounds were sensed by the antennae, rather than by hairs of the genital plaques.

KEY WORDS oviposition, artificial substrate, plant extracts

In a RECENT REVIEW ON plant chemical cues affecting oviposition by herbivorous insects, several references are made to species of Lepidoptera and Diptera, among other orders, but none was found regarding Heteroptera (Städler 2002). Understanding chemical mediators of oviposition in this important group of insects, which contains many agricultural pests, could be useful in the development of environmentally sustainable pest management.

Southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae), is a major pest of several crops worldwide, including soybean, *Glycine max* (L.) Merrill (Panizzi et al. 2000a). It feeds on plants of 30 families, such as Brassicaceae, Gramineae, Fabaceae, Rosaceae, and Solanaceae (Todd and Herzog 1980). It lays eggs in hexagonal-shaped masses, as do most stink bugs (Kiritani and Hokyo 1965, Javahery 1994). The number of eggs is variable (usually ≈80 eggs per mass), and eggs are laid mostly on the lower surface of the leaves of their host plants. Despite the many studies with this insect, summarized in Todd (1989), Panizzi (1997), and in Panizzi et al. (2000a), no reports were found regarding the influence of plant chemicals on its oviposition preference.

During many years of rearing this insect in the laboratory, we observed that the bugs frequently oviposit on the screen of cages. Also, *N. viridula* accepts paper towels suspended inside cages as oviposition substrates (Shearer and Jones 1996). Bugs also oviposit on artificial substrates, such as plastic structures mimicking soybean leaflets, exactly in the same way as they do on natural leaflets, by laying eggs on the lower (abaxial) surface (Panizzi et al. 2000b).

In this study, we developed a more rugged and reproducible artificial substrate and used it to test the effects of chemical extracts obtained from soybean plants (leaves and pods) on oviposition preference of *N. viridula*.

Materials and Methods

Plants. Plant of soybean 'Jack' were grown in the greenhouse, and leaves and pods were harvested from immature plants (pod-filling stage R6; Fehr et al. 1971). Immature soybean (R6) 'FS Rt 316' and 'FS Rt 3585' also were harvested from a field located in Peoria, IL, and taken to the laboratory.

[1] Embrapa-Labex-USA (Empresa Brasileira de Pesquisa Agropecuária-Laboratório no Exterior), Crop Bio-Protection, USDA–ARS, National Center for Agricultural Utilization Research, 1815 North University St., Peoria, IL 61604.
[2] Corresponding author, e-mail: panizzia@ncaur.usda.gov.
[3] New Crops and Processing Research, USDA–ARS, National Center for Agricultural Utilization Research, 1815 North University St., Peoria, IL 61604.
[4] Crop Bio-Protection, USDA–ARS, National Center for Agricultural Utilization Research, 1815 North University St., Peoria, IL 61604.

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Isolation of Compounds from Soybean Leaves and Pods. Leaves and pods were freeze-dried and ground to a fine powder by using a commercial coffee grinder. The powders (500 g of each) were extracted with hexane in a Soxhlet extractor overnight. The dried solid residue was refluxed with methanol in a Soxhlet extractor for 48 h. The remaining dried solid residue was extracted with water for 48 h at room temperature with stirring. The remaining solid material was removed by filtration and discarded. The hexane and methanol extracts were concentrated by rotoevaporation, and the water extract was concentrated by freeze drying. The remaining material in each fraction was resuspended in hexane, methanol, and water, respectively, for evaluation.

Chromatographic Separation of Methanol Extract from Immature Soybean Pods. The methanol extract from the soybean pods (39 g) was resuspended in 80% aqueous methanol and then evaporated or diluted with water to <5% methanol and loaded onto an equilibrated preparative C18 reverse phase (RP) column (45 by 6 cm, 125 Å; 55–105 μm; Waters, Milford, MA). The column was washed with water (≈700 ml, which is <1 column void volume) and eluted consecutively with 0, 20, 40, 60, 80, and 100% methanol in water (350 ml for each fraction). These fractions were labeled RP_0, RP_{20}, RP_{40}, RP_{60}, RP_{80}, and RP_{100}, respectively. The fractions were evaporated to dryness and resuspended (5–15 mg/ml) in methanol or a mixture of methanol and water for thin layer chromatographic (TLC) analysis and bioassay.

The most biologically active fraction from the C18 column, the RP_{20} fraction (23.5 g), was further fractionated by loading onto a silica gel column (S) (45 by 6 cm, grade 62, 60–200 mesh, 150 Å; Sigma-Aldrich, St. Louis, MO). The column was washed with 20% methanol in dichloromethane (≈700 ml, which is <1 column void volume) and eluted in a stepwise manner (350 ml) with 20, 40, 60, 80, and 100% methanol in dichloromethane. The 40 and 100% fractions did not yield detectable material. Only the 20, 60, and 80% fractions were evaluated, labeled as $RP_{20}S_{20}$, $RP_{20}S_{60}$, and $RP_{20}S_{80}$, respectively.

TLC Analysis. Selected fractions were spotted on TLC plates (silica gel 60 F 254, 20 by 20 cm, 2 mm in thickness; EM Scientific, Gibbenstown, NJ). The plates were developed with 10% methanol in dichloromethane. Phytochemicals were visualized by shortwave UV light and by spraying the dried developed plates with a solution of saturated potassium dichromate in concentrated sulfuric acid and heated to 130°C in an oven for 10 min. For the isolation of TLC fractions from the $RP_{20}S_{60}$ fraction (from a total yield of 15 g), preparatively spotted plates were allowed to dry, and sections containing spots visible under shortwave UV were scraped and extracted with methanol. The TLC fractions were concentrated by evaporation, and the dried material was resuspended in methanol for evaluation by bioassay.

Bioassays on Extracts and Chromatographic Fractions. From a rearing colony maintained in the laboratory at 25 ± 1°C, 65 ± 5% RH, and a photoperiod of 14:10 (L:D) h, 2-wk-old adults were separated and put (20 pairs) in each cage (45 by 45 by 25 cm) with green beans plus unshelled peanuts as food. Cheesecloth (Fisher Scientific Co., Pittsburgh, PA) was stretched tightly over wooden embroidery rings (10 cm in diameter) to receive the plant extracts to be tested. The hoops were placed on the floor of the cages and leaned against the cage walls, in a vertical position. Treatments were replaced approximately every 3 d.

Test 1. In this test, the following treatments were compared: hexane extract from leaves (HL), hexane extract from pods (HP), methanol extract from leaves (ML), methanol extract from pods (MP), and control (rings that were untreated, C). From each extract, 1 ml (containing ≈5–10 mg of extract) was applied on the cheesecloth of a ring by using a paint brush. Four rings were put inside each of four cages in random positions (two for each treatment), and the number of egg masses laid was compared as follows: HL versus C, ML versus C, HP versus C, MP versus C, HL versus ML, HL versus HP, ML versus MP, and HP versus MP. The number of egg masses deposited on each substrate was recorded daily. Each comparison lasted 7–12 d.

Test 2. In the second test, RP chromatographic fractions from MP were tested. The resuspended fractions were applied to the cheesecloth rings and placed in the cages as follows: cage 1, RP_0, RP_{40}, RP_{100}, and C (control); and cage 2, RP_{20}, RP_{60}, RP_{80}, and C (control). The number of egg masses laid on each ring was recorded daily for 7 d.

Test 3. In the third test, three of the silica column fractions obtained from the RP_{20} fraction ($RP_{20}S_{20}$, $RP_{20}S_{60}$, and $RP_{20}S_{80}$) were evaluated against C. Each fraction was applied on the cheesecloth as described previously, and the four rings were placed in one cage. The number of egg masses laid on each ring was recorded daily for 7 d.

Test 4. In the fourth test, TLC fractions that originated from the $RP_{20}S_{60}$ fraction (selected from the previous test) were evaluated. Four fractions were tested, using small cages (20 by 15 by 10 cm), each containing 10 pairs of 2-wk-old *N. viridula*. Five cages were used for each pair of treatments, and the numbers of egg masses were recorded daily during 4 d, totaling 20 observations per treatment. The following treatment combinations were compared: TLC_1 versus C, TLC_2 versus C, TLC_3 versus C, and TLC_4 versus C. In addition, the recombined TLC fractions (ΣTLCs) and the parent material ($RP_{20}S_{60}$) also were compared in pairwise manner: $RP_{20}S_{60}$ versus C, ΣTLCs versus C, and $RP_{20}S_{60}$ versus ΣTLCs.

Test 5. In the fifth test, the $RP_{20}S_{60}$ fraction was applied to just one half of an oval wooden embroidery ring (25 by 12 cm) covered with cheesecloth. The other half was untreated (control). Two rings were put in each of two cages, totaling four replications. The number of egg masses deposited on each half of the rings was recorded daily during 9 d.

Test 6. A final test was conducted with the $RP_{20}S_{60}$ fraction obtained from pods of green bean, *Phaseolus vulgaris* L. Two rings were placed inside a cage, one

and one untreated. The number of egg masses on each ring was recorded daily for 8 d.

Antennectomy. This study was conducted to test the role of the antennae in recognizing extracts applied on artificial substrates. Two cages (35 by 35 by 35 cm) each containing 10 pairs of 2-wk-old *N. viridula* were used. One cage received the antennectomized females, and the other received normal females (control). The $RP_{20}S_{60}$ fraction was applied on the cheesecloth, as described previously. Each cage contained two hoops, one treated and one untreated (control). The number of egg masses deposited on each hoop was recorded daily during 18 d.

Hairs on Genitalia. An additional test was carried out, similar to the one with antennectomized females, except that in this test females had the hairs on their genitalia coated with transparent nail polish. Data on the egg mass deposition on the hoops were recorded as described above.

Statistics. Pairs of treatments were usually compared by subjecting the numbers of egg masses to 1 df tests (H_0: treatments equivalent). However, test 1 and the final portion of test 4 were analyzed as incomplete two-dimensional contingency tables by using the Bradley–Terry paired comparison model (Fienberg 1977).

Results

Plant Extracts. Results from the bioassay to compare the hexane and methanol extracts from soybean leaves and pods (test 1) indicated that all extracts had a positive effect, with a much greater number of egg masses laid by *N. viridula* on the treated compared with the untreated artificial substrates (e.g., 4–30 times more masses). The MP extracts showed the strongest results, followed in order of decreasing activity by ML, HL, and HP (Table 1).

When MP extracts were subjected to reverse phase chromatography (test 2), the fractions eluted with water (RP_0) and 20% methanol in water (RP_{20}) received the greatest number of egg masses compared with controls. These two treatments yielded the highest values for the likelihood ratio statistics (G^2). The extracts with 40, 80, and 100% also produced significant responses, but the extract with 60% methanol in water (RP_{60}) did not differ from the control (Table 2).

When silica column fractions obtained from the RP_{20} fraction (selected from the previous test) were evaluated (test 3), the substrate containing the fraction $RP_{20}S_{60}$ elicited the greatest response. Although all three fractions tested were significantly better than the control in eliciting oviposition, the $RP_{20}S_{60}$ treatment received over twice as many eggs as the remaining two (Table 3). By weight, the $RP_{20}S_{60}$ fraction was 4% of the original dried soybean pods. Activity was detected using as little as 15 mg of this material per dish.

The $RP_{20}S_{60}$ fraction was spotted on TLC plates, and four fractions were obtained. When these were evaluated (test 4), only one fraction (TLC_1) showed a significantly greater ($P = 0.0162$) number of egg masses than the control, but it was less effective than the intact $RP_{20}S_{60}$ fraction (Table 4).

However, when all the TLC fractions were recombined (ΣTLCs), the positive oviposition effect occurred again, with the number of egg masses being significantly ($P < 0.001$) greater (7 × more) on the treated substrate compared with the control. When the ΣTLCs were compared with the original fraction ($RP_{20}S_{60}$), they were equally effective (Table 4).

The bioassay in which half of the area of the cheesecloth was treated with the $RP_{20}S_{60}$ extract and the

Table 1. Number of egg masses deposited by *N. viridula* on artificial substrates treated with soybean plant extracts, in a pairwise comparison

Treatment	Frequency[a]	Frequency ratio[b]	G^2 value[b]
HL	22 (23.0)	5.75	25.5***
C	5 (4.0)		
ML	24 (24.7)	10.74	45.7***
C	3 (2.3)		
HP	15 (13.1)	4.52	15.7***
C	1 (2.9)		
MP	29 (29.1)	32.33	105.7***
C	1 (0.9)		
HL	4 (5.5)	0.52	2.9 ns
ML	12 (10.6)		
HL	13 (10.5)	1.24	0.4 ns
HP	6 (8.5)		
HL	—[c]	0.17	18.9***
MP	—		
HP	—	0.42	4.5*
ML	—		
ML	9 (9.7)	0.32	13.8***
MP	31 (30.2)		
HP	5 (4.4)	0.14	35.0***
MP	32 (32.6)		

Abbreviations are defined in text. Values compared using likelihood ratio statistics (G^2) (test 1). * Significant difference ($P \leq 0.05$); *** significant difference ($P \leq 0.001$); ns, not significant.

[a] In parentheses, fitted values under the Bradley–Terry paired comparison model (model fits: $G^2 = 4.55$, df = 4, $P = 0.34$).

[b] Ratio calculated from the fitted frequency values.

[c] Direct experimental comparison of these treatments not done, but ratios could still be calculated from the Bradley–Terry model.

Table 2. Number of eggs masses deposited by *N. viridula* on artificial substrates using the RP chromatographic fractions (0, 20, 40, 60, 80, and 100% methanol in water) from the methanol extracts of pods

Treatment	Frequency	Frequency ratio (fraction/control)	G^2 value[a]
RP_0	60	30.00	68.3***
Control	2		
RP_{20}	72	6.54	50.1***
Control	11		
RP_{40}	19	9.50	15.9***
Control	2		
RP_{60}	14	1.27	0.4 ns
Control	11		
RP_{80}	32	2.91	10.7***
Control	11		
RP_{100}	37	18.50	38.3***
Control	2		

Values compared using likelihood ratio statistics (G^2) (test 2). *** Significant difference ($P \leq 0.001$); ns, not significant.

[a] df = 1.

Table 3. Number of eggs masses deposited by *N. viridula* on artificial substrates by using the reverse phase chromatographic fraction from the methanol extracts from pods (RP_{20}), further fractionated on a silica column (S)

Treatment (T)	Frequency	Frequency ratio (fraction/control)	G^2 value[a]
$RP_{20}S_{20}$	20	6.67	14.1***
Control	3		
$RP_{20}S_{60}$	46	15.33	45.4***
Control	3		
$RP_{20}S_{80}$	13	4.33	6.7**
Control	3		

Subscripts for silica fractions indicate the percentage of methanol in dichloromethane for the eluting solvent. Values compared using likelihood ratio statistics (G^2) (test 3). ** Significant difference ($P \leq 0.01$); *** Significant difference ($P \leq 0.001$).

[a] df = 1.

other half was untreated (test 5) showed that 100% of the egg masses recorded (total of 54 masses) were laid on the area that received the extract (four rings used in two cages).

An additional test conducted with the $RP_{20}S_{60}$ fraction obtained from green bean pods (test 6) indicated a significantly ($G^2 = 17.2$, P value <0.001) greater number of egg masses deposited on treated hoops (17) compared with the untreated control (1).

Antennectomy. Results of this bioassay demonstrated that antennectomized females (i.e., females that had their antennae removed) were not able to distinguish between the treated hoops with the soybean pod extract $RP_{20}S_{60}$ and the hoops that did not receive chemicals (control) (Table 5). In contrast, normal females distinguished between the two treatments and laid a significantly greater number of egg masses on the treated versus the control hoops.

Table 4. Numbers of eggs masses deposited by *N. viridula* on artificial substrates treated with thin layer chromatography fractions (TLC_{1-4}) derived from the $RP_{20}S_{60}$ fraction from the methanol extract of soybean pods and on controls

Treatment	Frequency	Frequency ratio	G^2 value[a]
TLC_1	12	4.00	5.8*
Control	3		
TLC_2	9	1.80	1.2 ns
Control	5		
TLC_3	7	1.17	0.1 ns
Control	6		
TLC_4	12	2.40	3.0 ns
Control	5		
$RP_{20}S_{60}$	19	5.84[b]	14.7***
Control	3		
ΣTLCs	14	7.27[b]	21.2***
Control	2		
$RP_{20}S_{60}$	12	0.80[b]	0.3 ns
ΣTLCs	9		

Recombined TLC fractions (ΣTLCs), TLC parent material ($RP_{20}S_{60}$), and controls also were compared in a pairwise test. Values compared using likelihood ratio statistics (G^2) (test 4). * Significant difference ($P \leq 0.05$); *** significant difference ($P \leq 0.001$); ns, not significant.

[a] df = 1.

[b] Frequency ratio based on fitted values from Bradley–Terry model for the three comparisons involving $RP_{20}S_{60}$, ΣTLCs, and control (model fits: $G^2 = 0.13$, df = 1, $P = 0.72$).

Table 5. Number of eggs masses deposited by *N. viridula* on artificial substrates treated with soybean plant fractions obtained using methanol from pods ($RP_{20}S_{60}$). Comparisons of females antennectomized (FANT), females with the hairs of the genitalia coated with nail polish (FHGC), and normal females (FNOR)

Treatment	Frequency	Frequency ratio	G^2 value[a]
FANT	9	1.5	0.6 ns
Control	6		
FNOR	40	20.0	42.1***
Control	2		
FHGC	13	6.5	9.0**
Control	2		
FNOR	22	7.3	16.3***
Control	3		

Values compared using likelihood ratio statistics (G^2). ** Significant difference ($P \leq 0.01$); *** significant difference ($P \leq 0.001$); ns, not significant.

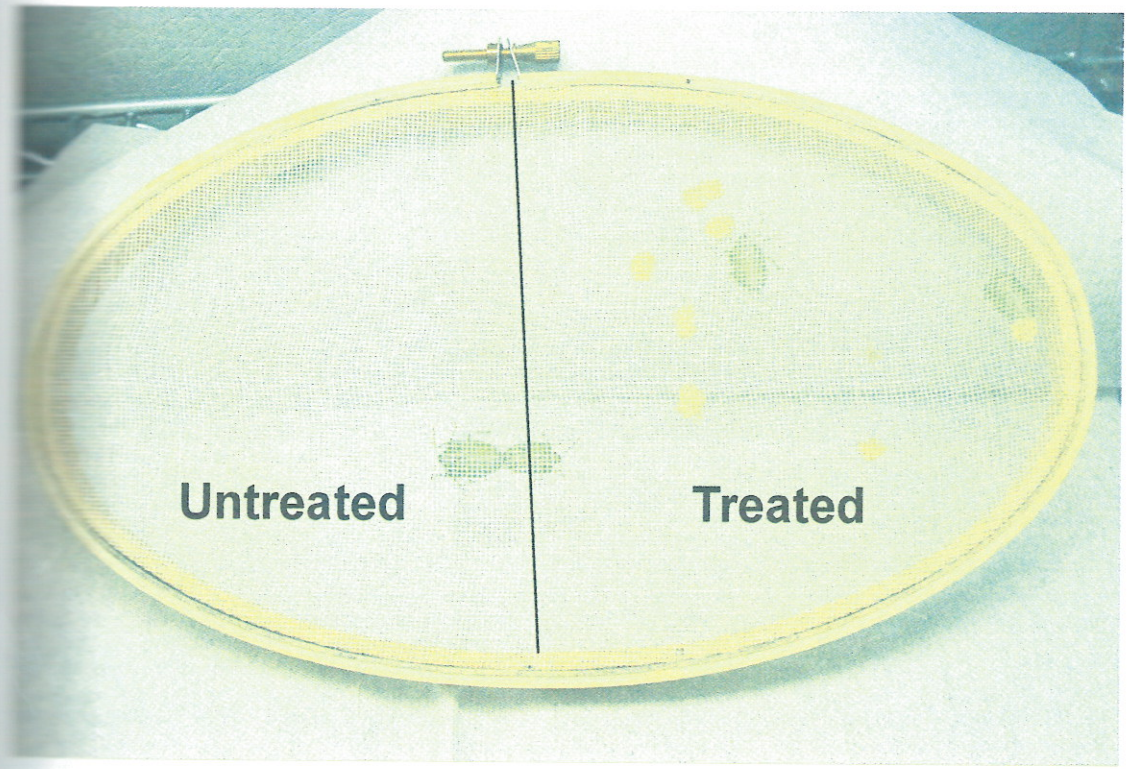
[a] df = 1.

Hairs on Genitalia. The preference of females to oviposit on hoops with fraction $RP_{20}S_{60}$ was not affected when the hairs of the genitalia were coated with nail polish (Table 5). However, they tended to lay a smaller number of egg masses than normal females.

Discussion

Results of these studies indicate that *N. viridula* females respond to soybean plant chemical extracts for egg deposition. The proclivity of females to oviposit exclusively on the half of the artificial substrate (cheesecloth) that was treated with a fraction from soybean pods ($RP_{20}S_{60}$), in contrast to the untreated area (Fig. 1), further supports the results of the bioassays that had treatments applied to separate hoops. Despite being extremely polyphagous, *N. viridula* displays a preference for feeding and ovipositing on plants within the Fabaceae and Brassicaceae (Todd and Herzog 1980). These results indicate females respond to specific chemicals contained in *G. max*, which is a legume. These chemicals or closely related ones, however, seem to be present in other species of legumes, such as green bean, as the results of test 6 demonstrated.

The fact that several extracts and chromatographic fractions obtained from leaves and pods of soybean had positive effects on *N. viridula* egg deposition in varying degrees may indicate that the chemical cues are made up of multiple components. In general, it is known that other oviposition stimulants that have been characterized consist of multiple components such as flavonoid glycosides, alkaloids, and cyclitols, as for swallowtail butterflies (Nishida 1995). Our results show that when the $RP_{20}S_{60}$ fraction obtained from soybean pods was further fractionated by TLC, the individual components were less active. However, when the TLC fractions were recombined (ΣTLCs), activity comparable with that of the original soybean pod $RP_{20}S_{60}$ fraction was regained. This may be a common pattern among insects. For example, gravid mosquitoes are attracted to oviposition sites by blends of compounds from Bermuda grass, rather than by



Egg masses of *N. viridula* laid on an artificial substrate (cheesecloth) treated with soybean pod fraction ($RP_{20}S_{60}$) with methanol. Note that all egg masses were deposited in the treated area rather than the untreated area.

chemicals (Du and Millar 1999). However, oviposition in response to a single host specompound is also common (Honda 1995).

studies on oviposition preferences of Hetera on host or nonhost plants have not examined chemical compounds that might influence such ces. For example, the alydid *Neomegalotomus* Westwood is known to oviposit in crevices of pigeon pea, *Cajanus cajan* L. These specific for egg laying are selected by mechanoreceptors in the ovipositor (Ventura and Panizzi 2000). another host plant, soybean, this bug oviposits on attaching the eggs near the midrib (Panizzi et . In these cases, hairs present on the tip of the itor are likely to be stimulated by the texture of substrates. With respect to southern green stink results of our bioassays demonstrate that the hairs on the ovipositor do not seem to be playing a oviposition, at least with respect to chemical ants. Although laboratory observations suggest females "evaluated" the substrate before ovipostouching it with the ovipositor before expelthe eggs, they might, at this time, be evaluating (e.g., texture) attributes. However, addistudies are needed to more clearly elucidate this

The fact that antennectomized females were unable locate the hoops treated with the soybean pod for oviposition and that females with antennae did indicated that antennal recognition of the chemical stimulants is involved.

In conclusion, results of these bioassays demonstrate that southern green stink bug selects artificial substrates treated with different chemical extracts obtained from the soybean plant to oviposit. The $RP_{20}S_{60}$ silica column fraction obtained from soybean pods was the most effective. Additional studies underway will be aimed at identifying the chemical compounds involved in this selection process.

Pheromones of *N. viridula*, in particular, and of stink bug species in general are much more chemically sophisticated than previously thought, and their practical applications in integrated pest management programs are far behind other insect orders (McBrien and Millar 1999). Our results on chemicals conditioning egg deposition by *N. viridula* on artificial substrate, open new possibilities of application of chemical ecology to manage this pest, such as enhancing the attractiveness of early planted trap crops or, in the laboratory, to induce oviposition on desired substrates for improved efficiency of egg collection to mass rear parasitoids. This and other strategies within this context should be investigated.

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