Volatiles Mediating a Plant-Herbivore-Natural Enemy Interaction in Resistant and Susceptible Soybean Cultivars

Mirian Fernandes Furtado Michereff · Raúl Alberto Laumann · Miguel Borges ·
Miguel Michereff-Filho · Ivone Rezende Diniz · Austeclínio Lopes Farias Neto ·
Maria Carolina Blassioli Moraes

Abstract Several studies have shown that herbivore-induced plant volatiles act directly on herbivores and indirectly on their natural enemies. However, little is known about the effect of herbivore damage on resistant and susceptible plant cultivars and its effect on their natural enemies. Thus, the aim of this study was to evaluate the attraction of the herbivorous pentatomid bug *Euschistus heros* and its egg parasitoid *Telenomus podisi* to two resistant and one susceptible soybean cultivars with different types of damage (herbivory, herbivory+oviposition, and oviposition). In a Y-tube olfactometer, the parasitoids were attracted to herbivory and herbivory+oviposition damaged soybean plants when compared to undamaged soybean plants for the resistant cultivars, but did not show preference for the susceptible cultivar Silvânia in any of the damage treatments. The plant volatiles emitted by oviposition-damaged plants in the three cultivars did not attract the egg parasitoid. In four-arm-olfactometer bioassays, *E. heros* females did not show preference for odors of damaged or undamaged soybean plants of the three cultivars studied. The Principal Response Curves (PRC) analysis showed consistent variability over time in the chemical profile of volatiles between treatments for the resistant cultivar Dowling. The compounds that most contributed to the divergence between damaged soybean plants compared to undamaged plants were (E,E)-α-farnesene, methyl salicylate, (Z)-3-hexenyl acetate, and (E)-2-octen-1-ol.

Key Words *Euschistus heros* · *Telenomus podisi* · Stink bug · Tritrophic interactions · Indirect defense

M. F. F. Michereff · R. A. Laumann · M. Borges ·
M. C. B. Moraes (✉)
Embrapa Recursos Genéticos e Biotecnologia,
Brasília, Brazil
e-mail: mcbmorae@cenargen.embrapa.br

M. F. F. Michereff · I. R. Diniz
Departamento de Zoologia, Universidade de Brasília,
Brasília, Brazil

M. Michereff-Filho
Embrapa Hortaliças,
Brasília, Brazil

A. L. Farias Neto
Embrapa Cerrados,
Brasília, Brazil

M. C. B. Moraes
Embrapa Recursos Genéticos e Biotecnologia-Parque Estação
Biológica-W5 Norte,
CEP 70770-900 Brasília, DF, Brazil

Introduction

Plants are equipped with several mechanisms to defend themselves against herbivores, microbial pathogens, or abiotic stress. One of the mechanisms of damage-induced plant defense is the production of plant volatile organic compounds (VOCs) that serve as repellents to herbivores or as attractants to parasitoids and predators (Price, 1997; De Moraes et al., 2001; Hilker and Meiners, 2006; Chen, 2008; Heil, 2008).

Plants change the blend of VOCs in response to damage either qualitatively or quantitatively (Dicke, 1999), depending mainly on plant traits that vary among species or genotypes and on the different herbivore species causing the damage (Lin et al., 2008; Rasmann and Turlings, 2008). This change can be responsible for differential attraction of parasitoids and predators (Fritzsche Hoballah and Turlings, 2001). Soybean plants, *Glycine max* L. (Fabales: Fabaceae)

and pigeon pea, *Cajanus cajan* (Valder) (Fabales: Fabaceae) damaged by *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae) released similar chemical profiles of volatiles that attracted the egg parasitoid *Telenomus podisi* (Ashmead) (Hymenoptera: Scelionidae). However, when soybean was damaged by feeding *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) larvae, the chemical profile was different and did not attract *T. podisi* (Moraes et al., 2005).

The identification of chemicals responsible for plant defense and their role in the interactions with natural enemies could be used to attract and retain parasitoids in crops (Gatehouse, 2002) and help breeders in the development of cultivars resistant to pests (Piubelli et al., 2005). Therefore, it is important that hostplant resistance interacts positively with the action of natural enemies. However, this association does not always result in positive interactions. Chemical and morphological plant traits can directly influence survival, fecundity, and foraging success of natural enemies on hosts or prey. These traits also can have indirect effects on the quality of herbivores that in turn affects the physiology, behavior, or development of their natural enemies (Cortesero et al., 2000). In a study investigating the effects of chemical plant resistance against herbivores and of predators on plant fitness, Bartlett (2008) observed a negative interaction between the predator *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) and soybean resistance against *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae).

Studies on resistance of soybean cultivars primarily involve direct defense against herbivory during the reproductive stage of soybean (Piubelli et al., 2005; Li et al., 2004, 2008). Carrão-Panizzi and Kitamura (1995) reported that the cultivar IAC100 contains high quantities of isoflavones compared to other soybean cultivars studied and showed high resistance to herbivorous stink bugs. The resistance of IAC100 to stink bugs and lepidopteran larvae was confirmed by McPherson and Buss (2007) and Piubelli et al. (2005) who proposed an antibiotic effect of this cultivar on velvetbean caterpillars, *A. gemmatalis*. The soybean cultivar Dowling shows antixenotic and antibiotic effects against aphids, which stop feeding and cause death after 48 hr (Li et al., 2004, 2008).

However, except for these studies of direct effects of soybean cultivar resistance on herbivores, no study has yet addressed the question how resistant traits of the vegetative stages of soybean cultivars affect the plant's indirect defense against herbivores. For Integrated Pest Management in soybean crops, it has been proposed that natural enemies could be released or attracted to the field before herbivorous stink bugs arrive (Borges et al., 1998). Therefore, it is important that the development of resistant cultivars does not alter plant responses to herbivore damage

and the release of herbivory-induced volatiles that attract natural enemies.

In this study, we tested the hypothesis that the level of indirect defense in response to damage by herbivores in resistant soybean cultivars is different from that of susceptible cultivars. Hence, we compared chemically the blend of herbivore-induced volatiles in resistant and susceptible cultivars during the vegetative stages. In addition, we compared the behavioral response of both herbivores and their parasitoids to induced volatiles released by the different cultivars. We chose two different cultivars that are known to display resistance against herbivores (IAC100 and Dowling) and one cultivar that shows high susceptibility to stink bugs (Silvânia) (Laumann et al., 2008). The studied herbivorous insect was the stinkbug *E. heros* and its egg parasitoid *T. podisi*.

Specifically, we studied the following questions: (a) Does *T. podisi* respond differently to resistant and susceptible cultivars when exposed to undamaged plants or plants damaged by herbivory and oviposition by *E. heros*? (b) Are *E. heros* females attracted to the volatiles of undamaged or damaged soybean cultivars? (c) Do resistant and susceptible soybean cultivars release a different chemical profile of volatiles when submitted to herbivory, herbivory+oviposition, or oviposition damage by *E. heros*? (d) Does attraction of *T. podisi* depend on the extent of herbivore damage?

Methods and Materials

Plant and Insect Rearing Soybean (cultivars Dowling, IAC100 and Silvânia) seeds were obtained from the Embrapa Cerrados Research Center (Brasília, DF, Brazil). Seeds were germinated on damp paper, and after 3 day they were transplanted to pots with a mixture of soil and organic substrate (in a proportion of 1:1 *w/w*). Plants were kept in a controlled environment room at L14:D10 photoperiod, $27\pm 1°C$ and $65\pm10\%$ r.h. All plants used were in the V3 physiological stage (Fehr et al., 1971).

Euschistus heros individuals were obtained from a laboratory colony started from adults collected in soybean fields near Embrapa Genetic Resources and Biotechnology, Brasília, Brazil (15°47′ S, 47°55′ W). Bugs were reared in 8 l plastic containers, on a diet of soybeans, sunflower seeds (*Helianthus annus*), raw peanuts (*Arachis hypogaea*), fresh green beans (*Phaseolus vulgaris*), and water. The food supply was renewed twice a week. To provide oviposition substrate and shelter for the bugs, a piece of a nylon mesh screen (15 cm^2) was placed inside the cages. To obtain virgin females, insects were sexed after their imaginal molt and cuticular hardening, and maintained isolated from males until their use in experiments.

The egg parasitoid *T. podisi* was obtained from a laboratory colony raised on eggs of *E. heros*. The wasps were maintained in acrylic cages (25 cm^2 tissue culture flask, angled neck; ICN Biomedicals, Irvine, CA, USA), and fed with a drop of honey, supplied twice a week. Once parasitoids hatched from eggs, they were transferred to an egg-free acrylic cage where they could mate for a period of 24 hr. *Euschistus heros* and *T. podisi* were maintained in separate environmental rooms at 27±1°C, 65±10% r.h., and a 14 hr photoperiod.

Preparation of Plants for Bioassays We studied the attraction of *T. podisi* to the three soybean cultivars (cv) that had been subjected to following treatments: (1) herbivory damaged soybean (Herb): five virgin, sexually immature *E. heros* females (2–4 day-old) were placed on the plant; (2) herbivory+oviposition damaged soybean (HerbOvi): five mated *E. heros* females (12–15 day-old) were placed on the plant; (3) oviposition damaged soybean (Ovi): five mated *E. heros* females, with their stylet removed using dissection scissors, were placed on the plant; (4) control: undamaged soybean (UD). For treatments, each plant was kept in a micropore plastic bag (bread bag) where the insects were introduced. Plants were used 24, 48, 72, 96, and 120 hr after damage, with 60 replicates for each time/treatment combination. All insects were removed from the plants a few minutes before the bioassays.

We studied the preference behavior of *E. heros* females by using soybean plants that had been submitted to the same treatments as described above. All bioassays were carried out using plants on the 4th d of damage, because preliminary results showed that natural enemies respond to damaged plants only on the 4th d, and the chemical analysis of the extracts collected from the air-entrainment also showed higher levels of volatiles released on the 4th d

Olfactometer Bioassays with Egg Parasitoids To determine if herbivory- or oviposition-induced volatiles have an effect on the foraging behavior of parasitoids, individual naïve female parasitoids were offered a choice between odors in a Y-shaped olfactometer. A square acrylic block (19×19 cm) with a Y-shaped cavity (1 cm thickness) sandwiched between two glass plates was used as the bioassay arena (Moraes et al., 2005). The trunk of the apparatus measured 8 cm, and each arm 7 cm. Damaged or undamaged plants were placed in glass chambers that were connected to the olfactometer via silicon tubing. Filtered (activated charcoal) and humidified air was pushed through the system at 0.6 l/min, and pulled through at 0.2 l/min, in a push-pull system. The behavior of the insect was monitored by a camera (CCD camera Sony SPT M324CE; Sony, Minato-Ku, Tokyo, Japan fitted with a 12.5–75.0 mm/F1.8 zoom lens)

coupled to SACAM software (Jorge et al., 2005) to register behavioral parameters. A single *T. podisi* female was introduced at the base of the Y-tube and observed for 600 sec, and the first choice in each arm was recorded by the software. The first choice was considered to have been made when the insect entered an arm and remained there for at least 20 sec. Each individual was used only once, and the plants were replaced after every five repetitions. The position of the arms of the olfactometer was changed after every five bioassays in order to avoid any bias in the parasitoid response. The response of the parasitoid females was tested for each combination as follows: (1) Herb vs. UD (*N*=60); (2) HerbOvi vs. UD (*N*=60), and (3) Ovi vs. UD (*N*=60). The bioassays were carried out for each soybean cultivar 24, 48, 72, 96, and 120 hr after the start of damage. The damaged and undamaged plants were kept under the same conditions, but in different rooms to avoid plant–plant signalling, until used in the bioassays.

Olfactometer Bioassays with Herbivore Females A four-arm-olfactometer modified from Vet et al. (1983) was used with *E. heros* females. The olfactometer consisted of a square acrylic block (44×44 cm) with an X-shaped cavity (1.5 cm) sandwiched between two glass plates, and it was used as the bioassay arena. The "X" of the apparatus measured 37 cm, and length of the active area for each arm was 25 cm. The length of each arm considered for a positive response of the insects was 15 cm, measured from the upwind end towards the center of the olfactometer. The bioassay procedure was the same as described for the Y-olfactometer above. The first choice was considered to have been made when the insect entered an arm and remained there for at least 60 sec. The responses of 70 stink bug females were tested for each combination: (1) Herb vs. HerbOvi vs. Ovi vs. UD for each soybean cultivar (cv) with 96 hr of damage and (2) UD of cv Dowling vs. UD cv IAC100 vs. UD cv Silvânia vs. air. For each combination, we tested females in different physiological conditions: (a) starved virgin females (the diet was removed 24 hr before the bioassay); (b) fed virgin females; and (c) fed mated females.

Volatile Analysis Herb-, HerbOvi-, Ovi-, and UD-plants were placed individually in cylindrical glass chambers (internal volume 10 l). Volatiles were collected for 24 hr, during seven consecutive days (*N*=11 replicates for each treatment). In an attempt to minimize contamination by volatiles from the soil, pots were wrapped in aluminium foil. A glass tube containing the adsorbent Super Q (100 mg, 80-100mesh, Alltech PA, USA) was connected via a PTFE tube to a vacuum pump at a flow of 0.6 l/min, and the air entrance was connected to a flow of air filtered with activated charcoal (1.0 l/min) creating a positive push-

pull system (Moraes et al., 2008). The trapped volatiles were eluted from the adsorbent using 500 μl n-hexane and pre-concentrated to 200 μl under a N_2 flow. Extracts were stored at −20°C until analysis by gas chromatography (GC) and GC coupled to mass-spectrometry (GC-MS).

The extracts obtained by air entrainment were analyzed by GC (Shimadzu 17A, DB-5 column, 30 m×0.25 mm ID, 0.25 μm film, J&W Scientific, Folsom, CA, USA), using a temperature program of 50°C/1 min, 15°C/min to 250°C/ 20 min. The column effluent was analyzed with a flame ionization detector (FID) at 270°C. For the GC analyses, 50 μl of each extract were separated, and 1 μl of 1-octen-3-ol was added as internal standard (IS), for a final concentration of 9.8 μg/ml of the IS. One microliter of each sample was injected in splitless mode with helium as carrier gas. Amounts released by the plant in each 24 hr period were calculated in relation to the area of the internal standard. Data were collected with ClassGC software and were handled using Excel Software (Microsoft Corporation 2003).

For qualitative analysis, selected extracts were analyzed using a Shimadzu QP2010 instrument equipped with a quadrupole analyzer, a non-polar DB-5 column (30 m× 0.25 mm ID, 0.25 μm film, J&W Scientific, Folsom, CA, USA) and a splitless injector, with helium as the carrier gas. Ionization was by electron impact (70-eV, source temperature 200°C). Data were collected and analyzed with GC-MS Solutions 2.4 Software (Shimadzu Corporation, Japan). Identifications were made by comparison of spectra with library databases (NIST 2005) or with published spectra and confirmed by using authentic standards when available.

Chemicals Super Q (80/100 mesh) was purchased from Alltech (PA, USA). n-Hexane (95% pesticide residue) was purchased from Fisher Scientific (Loughborough, Leicestershire, UK). (E)-2-Hexenal and (Z)-3-hexen-1-ol were purchased from Sigma Aldrich (Gillinghan, Dorset, UK). (Z)-3-Hexenyl acetate was purchased from Alfa Aesar (Heysham, UK) and (E)-ocimene from Botanix (Paddock Wood, Kent, UK). Limonene and linalool were purchased from TCI America (Portland, OR, USA). Methyl salicylate, β-caryophyllene, benzothiazole, β-myrcene, geranyl acetone, and (E)-2-hexenyl acetate were purchased from Sigma Aldrich (Steinheim, Germany). (E,E)-α-Farnesene was provided by Jeffrey Aldrich (USDA-ARS, USA) and (E)-4,8-dimethylnona-1,3,7-triene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) were provided by Michael Birkett (Rothamsted Research, UK).

Statistical Analysis The null hypothesis of equal preference ($P=0.05$) of parasitoids for the volatiles from damaged plants vs. undamaged plants was tested at each sampling time (24, 48, 72, 96, and 120 hr), using *chi-square* tests with the Yates correction for continuity (Zar, 1998).

Since the volatile sampling was carried out on the same plant at several time points, repeated measures analysis of variance was used to avoid the problem of pseudo-replication during the times analyzed (Hurlbert, 1984; Green, 1993). Therefore, the total amounts of volatiles released in each treatment over time were compared by MANOVA (PROC ANOVA with the PROFILE statement; SAS Institute, 2001), using the subsequent sampling times as repeated measures of the same experimental unit, as suggested by von Ende (1993). Significant data by MANOVA were subjected to *post-hoc* mean comparisons in individual sampling time using the Tukey's test (significance level 5%).

The change in the chemical profile of damaged and undamaged soybean plants over time was assessed using Principal Response Curves (PRC) analysis. PRC models the effect of the treatment d at a determined time t in a species k. The statistical model for compound concentration is: ($y_{d(j)tk}=y_{0tk}+b_k\ c_{dt}+\varepsilon_{d(j)tk}$), where, $y_{d(j)tk}$ is the concentration of the compound k in replicate j of treatment d at time t, y_{0tk} is the mean concentration of the compound k in time t in the control (untreated plant, $d=0$), c_{dt} is a basic response pattern for every treatment d, and time t, b_k is the weight of each compound with this basic response pattern, and $\varepsilon_{d(j)tk}$ is an error term with mean zero and variance σ_k^2. These parameters were generated through redundancy analysis (RDA) least-squares estimates from reduced-rank regression with concomitant variables (van den Brink and ter Braak, 1999), with $N=11$ replicates for each sampling time and treatment.

The values of c_{dt} for the treatment (treatment effect) were plotted as vertical axis (first principal component, PRC1) over sampling time generating a PRC diagram, with a curve for each treatment, that describes the dynamic changes in the blend of volatiles released by the plant. We compared the variation in volatile composition in damaged and undamaged soybean, the latter being our standard ($c_{dt}=0$). The response variable weights (displayed on the right side of each diagram) allow evaluation of the contribution of each compound to the overall blend composition. The higher the values of the weight of the variable, the higher the contribution of the compound to the actual response pattern of the PRC.

PRC analysis was applied separately to each soybean cultivar, comparing the damaged and undamaged soybean. For each set of analyses, the significance of each PRC diagram was tested using an F-type test obtained by permutating (Monte Carlo permutation tests; 999 permutations) whole time series in the partial RDA from which the PRC was obtained. A second series of permutation tests (individual contrasts) was performed within each sampling date to determine if differences indicated by the PRC diagram between treatments were statistically significant.

These analyses were carried out using the software CANOCO 4.5 for Windows (ter Braak and Šmilauer, 2002). The data from the main volatile compounds responsible for the treatment differences in PRC diagram were individually submitted to repeated measures MANOVA followed by contrasts formed within sampling time to compare induction treatments against the control, using the t test (significance level 5%).

Results

Olfactometer Bioassays with Egg Parasitoids When testing the resistant soybean cultivar Dowling, the egg parasitoid *T. podisi* responded preferentially to the odor of plants with herbivory (Herb; $\chi^2=7.67$, $P=0.005$; $\chi^2=8.06$, $P=0.005$) and herbivory plus egg deposition (HerbOvi; $\chi^2=9.60$, $P=0.002$; $\chi^2=8.06$, $P=0.005$) when compared to undamaged plants (UD) after a 72 and 96 hr treatment-period, respectively (Fig. 1). *Telenomus podisi* preferred volatiles from the resistant soybean cultivar IAC100 when these have been treated for 96 hr by herbivory (Herb, $\chi^2=11.26$, $P=0.001$) and herbivory plus egg deposition (HerbOvi, $\chi^2=8.06$, $P=0.005$) to volatiles from undamaged (UD) plants. No preference was observed to the odors of cv IAC100 with other treatments at the different times evaluated (Fig. 2). For the susceptible cultivar Silvânia, *T. podisi* did not discriminate between odors emitted by undamaged plants and damaged plants regardless of the treatments and duration of treatment ($P>0.05$) (Fig. 3).

Olfactometer Bioassays with Herbivore Females In the four-arm-olfactometer bioassays, *E. heros* females did not show a preference for the Herb-, HerbOvi-, Ovi-, or UD-plants (χ^2, $P>0.05$) of any the three cultivars Silvânia, IAC100 and Dowling, or for undamaged plants of the three cultivars tested against air. This result was independent of the physiological state of the females (virgin starved, virgin fed, and mated fed).

Volatile Analysis The total amount of volatiles released by the soybean cultivar Dowling differed between treatments throughout the sampling times (treatment: $F=9.89$; $d.f.=3$, 56; $P=0.001$; interaction: *Wilk's* $\lambda=0.383$; $d.f.=18$, 145; $F=3.25$; $P=0.001$) (Fig. 4). The chemical profile of Dowling plants treated by herbivory (Herb), herbivory plus egg deposition (HerbOvi), egg deposition only (Ovi) differed when compared to undamaged plants (UD) at 48 hr of treatment and at longer treatment periods (*post-hoc* pairwise comparisons by Tukey test, $P<0.05$). No difference was observed for treatment effects or its interaction with time for the cv IAC100 (treatment: $F=1.82$; $d.f.=3$, 56; $P=0.15$; interaction: *Wilk's* $\lambda=0.583$; $d.f.=18$, 145; $F=1.61$; $P=0.06$) or cv Silvânia (treatment: $F=2.63$; $d.f.=3$, 56; $P=0.06$; interaction: *Wilk's* $\lambda=0.607$; $d.f.=18$, 145; $F=1.55$; $P=0.08$) (Fig. 4).

The chemical profile of the extracts obtained from the treatments Herb, HerbOvi, Ovi, and UD differed between the three cultivars Dowling, IAC100, and Silvânia. The compounds (Z)-2-octen-1-ol and (E)-2-octen-1-ol were identified only in cv Dowling; (E)-2-hexenyl acetate, and indole were identified in cv IAC100, while DMNT was
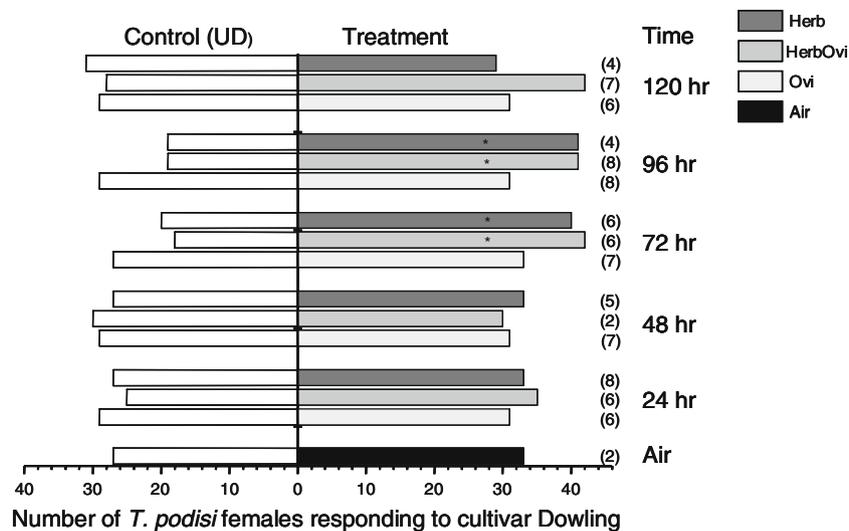
Fig. 1 Responses of the egg parasitoid *Telenomus podisi* in a Y-tube olfactometer to volatiles from soybean cultivar Dowling subjected to different treatments. *UD* undamaged soybean, *Ovi* soybean plant with oviposition damage, *HerbOvi* soybean plant with herbivory +oviposition damage, *Herb* soybean plant with herbivory damage. 24, 48, 72, 96 and 120 hr: time of plant treatment (induction time). *Asterisks* indicate significant differences ($P<0.05$) between parasitoid responses to volatiles from different treatments: χ^2 test on the proportion responding. *Numbers in brackets* represent the number of insects that did not respond to either of the treatments tested

Fig. 2 Responses of the egg parasitoid *Telenomus podisi* in a Y-tube olfactometer to volatiles from soybean cultivar IAC100 subjected to different treatments. *UD* undamaged soybean, *Ovi* soybean plant with oviposition damage, *HerbOvi* soybean plant with herbivory+oviposition damage, *Herb* soybean plant with herbivory damage. 24, 48, 72, 96 and 120 hr: time of plant treatment (induction time). *Asterisks* indicate significant differences (P<0.05) between parasitoid responses to volatiles from different treatments: χ² test on the proportion responding. *Numbers in brackets* represent the number of insects that did not respond to either of the treatments tested
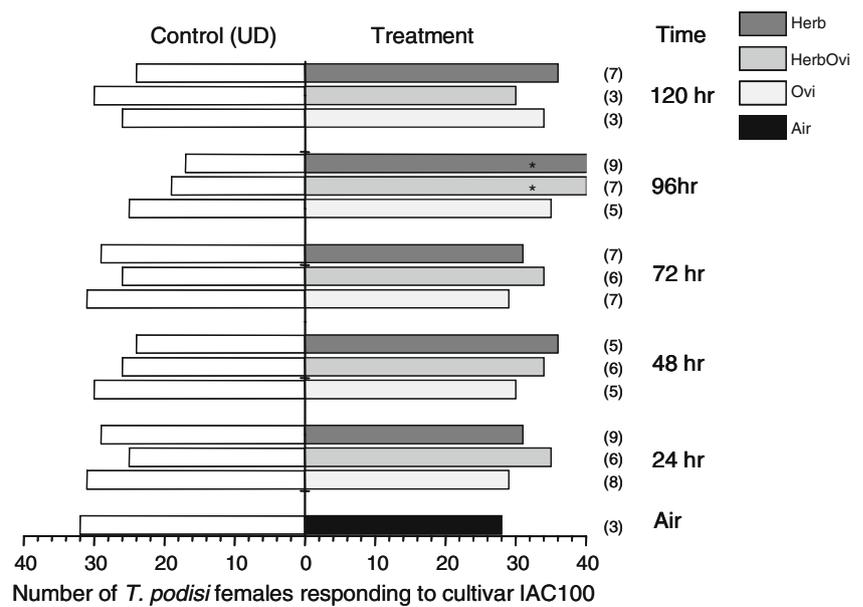
identified only in cv Silvânia (Table 1). The cultivars also emitted common compounds, such as the green leaf volatile (Z)-3-hexenyl acetate; the terpenoids camphene, limonene, α-humulene, β-caryophyllene, (E,E)-α-farnesene, TMTT, and the shikimic acid derivatives benzothiazole, benzaldehyde, and methyl salicylate. In addition, some compounds were released in higher quantities by damaged soybean when compared to undamaged soybean (Table 1).

Soybean Cultivar Dowling The PRC analysis of the plant volatiles released showed that cv Dowling had a consistent

variability over time between treatments. Of the total variance in the blend composition of volatiles released, 8.6% is explained by sampling times and 12% is explained by induction treatments. The variance exhibited in the first PRC axis was highly significant (Monte Carlo permutation test F=35.0, P=0.001), and explained 63.0% of the variation in the blend composition due to interactions between sampling times and induction treatments (Fig. 5). At 24 hr after damage, the analysis did not show a significant difference, indicating that the blends of the four treatments were similar. From 48 hr onward, PRC showed a
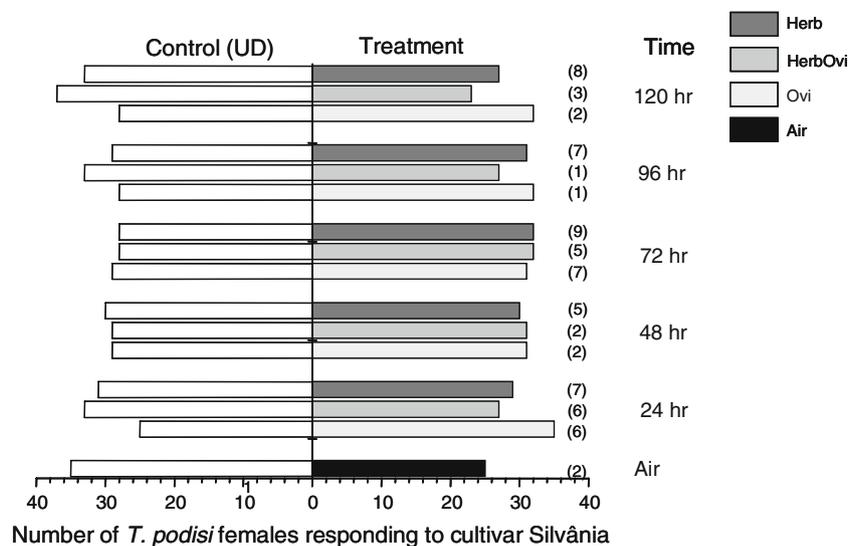
Fig. 3 Responses of the egg parasitoid *Telenomus podisi* in a Y-tube olfactometer to volatiles from soybean cultivar Silvânia subjected to different treatments. *UD* undamaged soybean, *Ovi* soybean plant with oviposition damage, *HerbOvi* soybean plant with herbivory +oviposition damage, *Herb* soybean plant with herbivory damage. 24, 48, 72, 96 and 120 hr: time of plant treatment (induction time). *Asterisks* indicate significant differences (P<0.05) between parasitoid responses to volatiles from different treatments: χ² test on the proportion responding. *Numbers in brackets* represent the number of insects that did not respond to either of the treatments tested
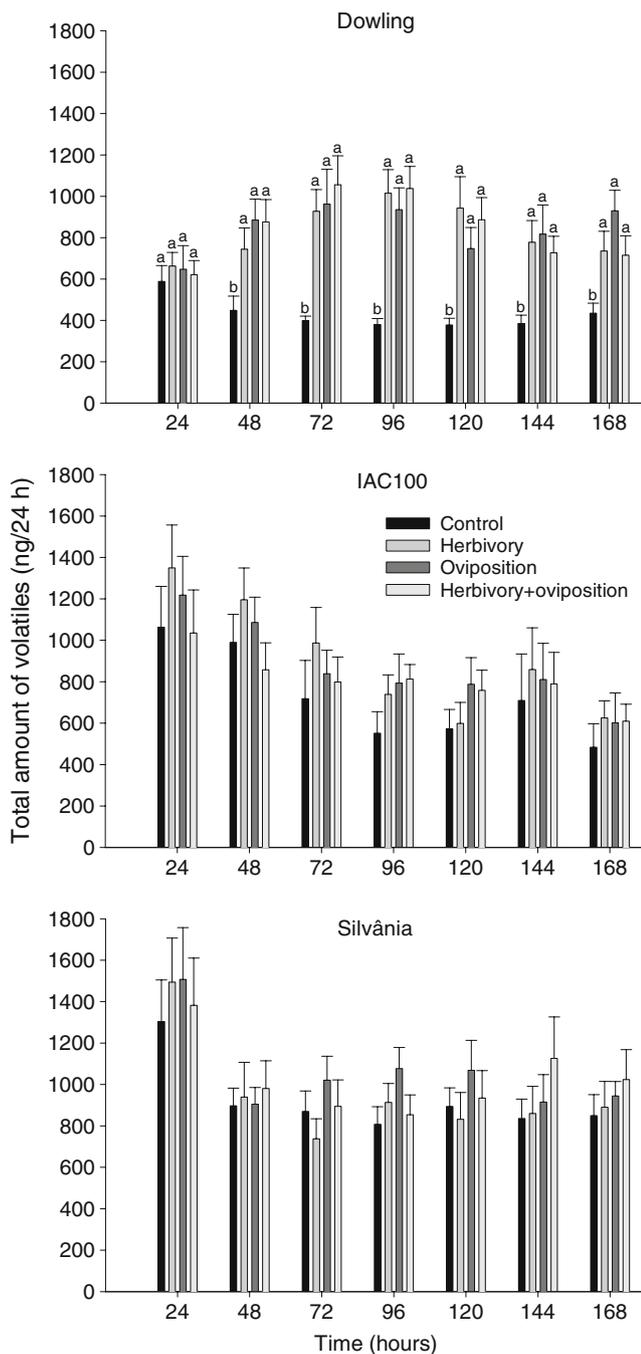
Fig. 4 Total amount of volatiles (mean + SEM) obtained from undamaged plants (control) and plants submitted to the treatments herbivory, oviposition, and herbivory+oviposition for soybean cultivars Dowling, IAC100, and Silvânia. The increase in the amount of volatiles released by damaged plants of cultivar Dowling was significant after 48 hr. For cultivars IAC100 and Silvânia there was no significant difference between treatments and its interactions with time (MANOVA for repeated measures, $P>0.05$). Means followed by the same letter within each sampling date are not significantly different by the Tukey's test at $P>0.05$

clear divergence in curves of the treatments when compared to the undamaged soybean (Fig. 5).

All variable weights (b_k) showed on the right side of the PRC diagram were positive (Fig. 5), which means that all compounds were positively associated with the pattern of changes in the volatile blend. Compounds with weight values higher than 0.5 contribute to the overall volatile blend response (van den Brink and ter Braak, 1999) and the greater the b_k value, the higher the influence on the blend response. Thus, (E,E)-α-farnesene, methyl salicylate, (Z)-3-hexenyl acetate, and (E)-2-octen-1-ol were the main compounds that contributed (b_k absolute values >1.0) to the divergence between treatments and control for the cv Dowling (Fig. 5).

A significant interaction between treatment and time (MANOVA for repeated measures, $P<0.05$) was found when data for the main compounds of cultivar Dowling were analyzed individually (Fig. 6). Plants with the treatments Herb and HerbOvi differed from UD plants (contrasts within sampling date; t test, $P<0.05$), releasing significantly higher quantities of the same compounds as indicated by PRC analysis (Fig. 6).

Soybean Cultivars IAC100 and Silvânia No significant treatment effect on the volatile blend composition was observed in PRC analysis for the cultivars IAC100 ($F=1.9$, $P=0.073$; Fig. 7, Table 1) and Silvânia ($F=10.4$, $P=0.104$; Fig. 8, Table 1). The variable weights (b_k) displayed in the PRC diagrams showed low values, ranging between −0.5 and 0.5, for all compounds sampled (Figs. 7 and 8).

Although no differences were observed by PRC for IAC100, some compounds were released in higher quantities by plants with the treatments Herb and HerbOvi when compared to UD and Ovi (Table 1). When we analyzed the compounds individually (MANOVA for repeated measures, $P<0.05$), (Z)-3-hexenyl acetate ($t_{59,3}=2.031$, $P=0.055$), methyl salicylate ($t_{59,3}=2.159$, $P=0.035$), and (E,E)-α-farnesene ($t_{48,3}=1.909$, $P=0.061$) were released in significantly or marginally non-significantly higher amounts from plants subjected to 72-hr herbivory (Herb) when compared to undamaged plants (UD). (Z)-3-Hexenyl acetate ($t_{59,3}=2.071$, $P=0.043$), methyl salicylate ($t_{59,3}=1.834$, $P=0.072$) and (E,E) α-farnesene ($t_{55,3}=2.820$, $P=0.006$) were released in significantly or marginally non significantly higher amounts from plants subjected to 96 hr herbivory-plus-oviposition (HerbOvi) when compared to undamaged ones (UD). (E,E)-α-farnesene ($t_{55,3}=2.322$, $P=0.023$) was released in significantly higher amounts from plants after 96 hr herbivory (Herb) when compared to undamaged plants (UD). The quantities of compounds released by Silvânia also were analyzed by MANOVA for repeated measures, but they did not differ among treatments.

Table 1 Volatiles from differently treated soybean cultivars Dowling, IAC100, and Silvânia

Compounds	Amounts of volatiles [ng 72–96 hr][a]			
	Treatment[b] UD	Herb	Ovi	HerbOvip
Dowling				
Camphene	14.8±2.4	17.9±3.8	37.1±9.6	26.4±8.2
Benzaldehyde	13.6±2.9	20.5±4.2	18.4±5.1	27.4±9.9
(Z)-3-Hexenyl acetate	92.5±19.0a	226.4±64.9b	185.0±39.2a	278.6±51.4b
Limonene	31.2±6.8	41.1±8.0	62.9±11.9	48.0±9.5
(Z)-2-Octen-1-ol[c]	20.2±2.0a	39.4±10.9a	85.4±16.9b	70.7±17.2b
(E)-2-Octen-1-ol[c]	50.6±8.5a	117.3±21.4b	218.9±52.9b	204.8±60.6b
Methyl salicylate	115.3±8.4a	437.8±43.8b	240.8±39.5b	462.6±61.4b
Benzothiazole	13.4±2.4	24.6±3.6	35.7±14.1	15.0±2.6
(E)-2-Decenal	9.1±3.4	5.5±1.3	17.9±4.4	16.7±4.8
β-Caryophyllene	30.2±8.5	54.2±13.2	57.8±12.9	50.6±11.5
α-Humulene	42.6±7.0	52.0±10.6	92.1±22.0	85.6±16.9
(E,E)-α-Farnesene	213.8±24.0a	623.4±75.1b	526.2±74.8b	562.2±66.9b
TMTT	55.9±10.7	114.0±36.8	94.2±25.9	90.9±22.9
IAC100				
(E)-2-Hexenal	16.6±7.9	32.0±18.6	12.2±6.3	39.1±18.1
(E)-2-Hexen-1-ol	24.7±10.4	12.2±4.4	15.1±6.4	13.5±4.4
(Z)-3-Hexen-1-ol	29.8±18.8	10.6±2.6	20.3±12.5	10.7±2.4
Camphene	36.7±13.4	48.4±22.2	47.6±16.9	29.1±11.5
Benzaldehyde	37.2±18.1	19.1±4.7	32.1±12.8	19.9±4.2
(Z)-3-Hexenyl acetate	40.8±16.3a	123.3±52.6b	73.3±19.0a	97.2±30.9b
(E)-2-Hexenyl acetate	22.7±16.8	52.8±34.9	26.4±11.9	17.2±1.7
Limonene	87.7±37.9	54.5±13.3	110.6±31.8	64.4±22.5
(E)-Ocimene	51.1±13.3	65.8±15.6	102.3±60.4	112.5±18.3
Linalool	64.5±18.4	94.0±22.9	166.3±58.8	118.9±22.7
Methyl salicylate	36.6±15.2a	123.9±9.8b	78.9±30.8a	135.1±50.1b
Benzothiazole	15.7±4.7	21.3±42.6	14.5±4.3	16.7±4.2
Indole	20.0±8.2	12.6±3.7	21.0±8.2	19.7±4.2
β-Caryophyllene	101.9±43.5	89.2±5.0	83.6±21.3	90.3±22.3
Geranyl acetone	107.7±36.5	91.3±28.3	122.5±27.2	105.8±26.8
α-Humulene	271.9±68.7	126.9±45.3	198.4±65.4	279.2±77.2
(E,E)-α-Farnesene	149.5±42.7a	362.9±86.8b	247.9±64.3a	354.2±62.4b
TMTT	67.3±24.1	82.9±18.9	84.1±20.3	70.3±17.9
Silvânia				
(E)-2-Hexenal	26.1±12.1	52.7±19.8	64.7±23.6	47.6±18.7
(E)-2-Hexen-1-ol	35.7±7.6	20.9±6.0	41.2±11.4	45.5±13.1
(Z)-3-Hexen-1-ol	26.2±7.1	20.8±9.8	42.4±12.8	32.8±10.2
Camphene	42.9±6.1	83.2±36.2	79.6±24.9	46.5±7.7
Benzaldehyde	21.0±4.3	19.0±2.7	50.2±11.3	48.0±14.2
(Z)-3-Hexenyl acetate	86.2±19.4	52.1±22.9	75.6±24.5	76.4±30.9
Limonene	74.4±16.9	96.9±18.6	175.3±38.8	137.2±28.6
(E)-Ocimene	105.5±22.2	119.7±26.1	179.9±18.9	152.9±32.9
Linalool	134.7 ±20.6	144.9±24.0	169.3±27.9	141.5±21.3
DMNT	41.5±11.7	46.2±11.1	61.0±9.9	44.0±9.2
Methyl salicylate	30.1±8.8	46.3±11.8	45.9±11.3	46.5±11.6
Benzothiazole	13.9±2.9	19.4±6.3	13.4±3.5	23.7±8.9
β-Caryophyllene	31.3±9.2	92.1±37.2	41.5±13.1	42.7±18.2

Table 1 (continued)

Compounds	Amounts of volatiles [ng 72–96hr][a]			
	Treatment[b] UD	Herb	Ovi	HerbOvip
Geranyl acetone	138.8±30.8	164.5±29.2	167.1±38.5	138.6±38.3
α-Humulene	140.0±27.3	192.6±39.8	226.0±62.9	219.7±53.0
(E,E)-α-Farnesene	153.8±25.1	123.3±19.2	222.8±46.5	201.8±44.4
TMTT	66.7±15.8	44.6±15.1	72.4±19.4	49.8±17.4

[a] Mean values (± SEM) calculated from 22 samples obtained by aerations during the period spanning 72-96 hr after the start of damage

[b] (UD) undamaged plants; or plants submitted to treatments Herbivory (Herb), Oviposition (Ovi) and Herbivory+Oviposition (HerbOvi) damage. Means followed by the same letter within a line are not significantly different by the MANOVA at $P > 0.05$. TMTT ((E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene), DMNT ((E)-4,8-dimethylnona-1,3,7-triene)

[c] Tentatively identified compounds

Discussion

In this study, by comparing the susceptible cultivar Silvânia and the resistant cultivars Dowling and IAC100, it is apparent that each cultivar has a different response to herbivore damage, eliciting different responses by the egg parasitoid. The results support our hypothesis that the level of indirect defense in response to damage by herbivores in resistant soybean cultivars is different from that of susceptible cultivars. We could show that the resistant cultivars (here Dowling and IAC100) showed stronger induction of indirect defense; they show a higher production of volatile compounds in response to herbivore attack and a stronger response by the egg parasitoid, *T. podisi*, to volatiles released from plants with herbivory and herbivory+oviposition when compared to undamaged plants. In addition, the results obtained in this study agree with those presented by previous work on induced plant responses to oviposition by Pentatomidae (Colazza et al., 2004a, b; Moraes et al., 2008), where oviposition itself (without herbivory) did not induce changes of plant odors that are attractive to the egg parasitoids.

However, egg-laying by the pentatomid *E. heros* on the cultivars Dowling and IAC100 changes the plant volatile profile; we have detected differences in the
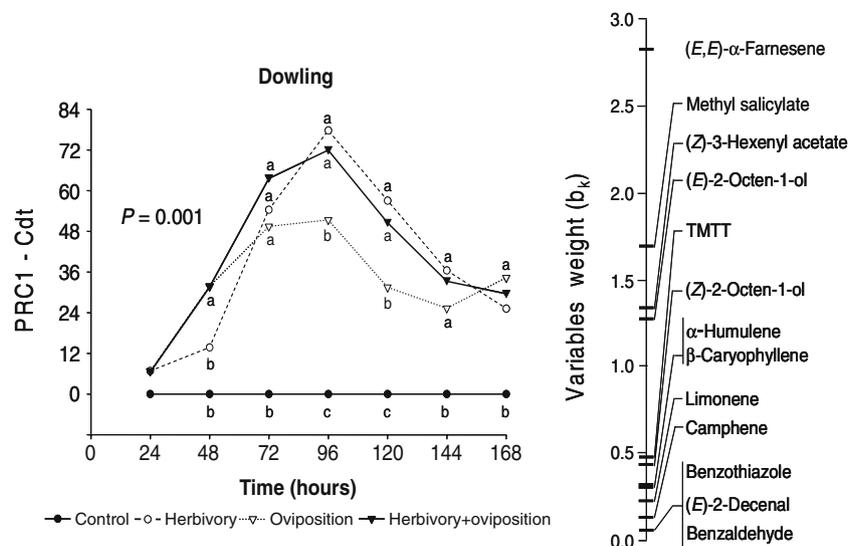
Fig. 5 PRC diagram and variables weights (b_k) based on volatile blends released by soybean plants of the cultivar Dowling, on seven sampling days. The lines represent the response pattern of soybean plants to different treatments in time. The *P*-value indicates significance of the PRC diagram over all sampling times based on *F*-type permutation test. On each date, values of C_{dt} differ significantly between treatments that do not share the same letter code (*a–c*); shared or omitted letter codes denote contrasts that do not differ significantly by the Monte Carlo permutation tests at $P < 0.05$. The higher (absolute values) the variable weight, the more closely the compound response pattern follows the deviation pattern (from the control, $C_{dt} = 0$ line) indicated on the PRC
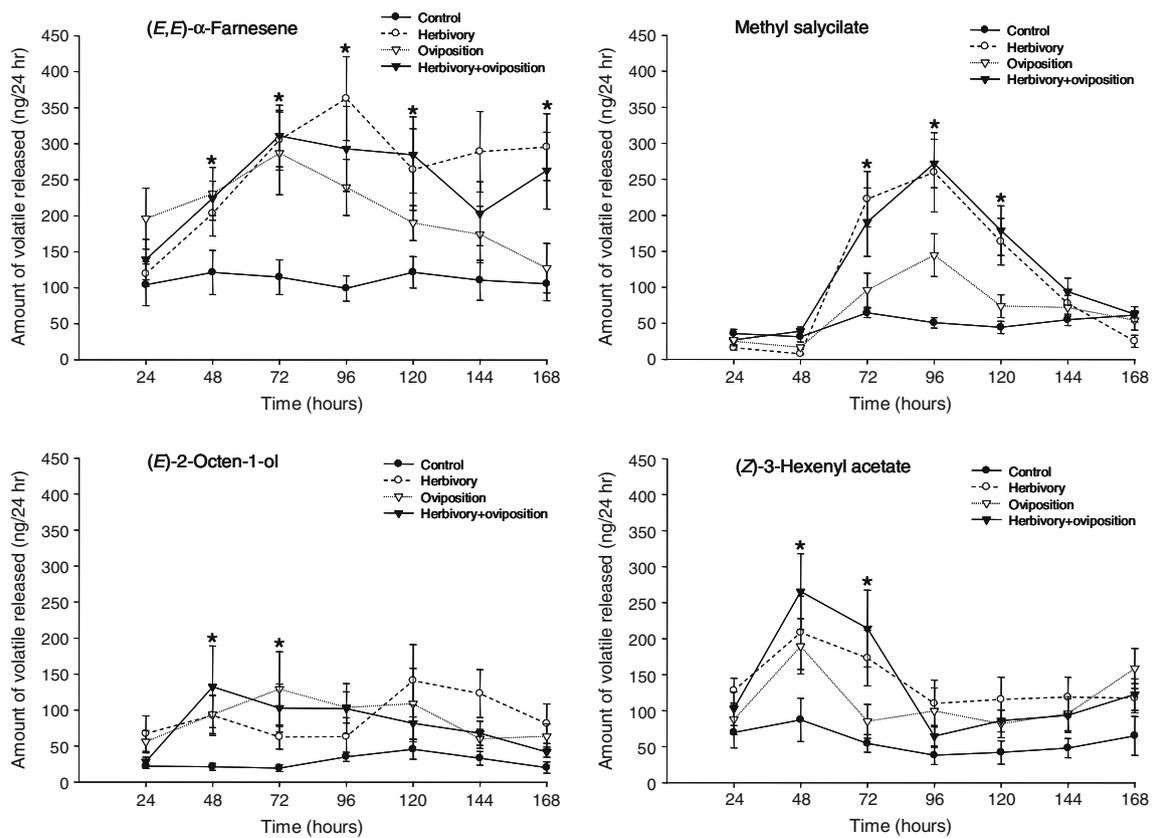
Fig. 6 Amounts (mean ± SEM) of principal volatile compounds released by undamaged soybean (control) and soybean plants submitted to herbivory, oviposition and herbivory+oviposition damage, for cultivar Dowling. *Bars* represent the standard error of the mean. Means marked with an *asterisk* indicate significant differences ($P<0.05$) between induction treatments and control (within a sampling date), t test
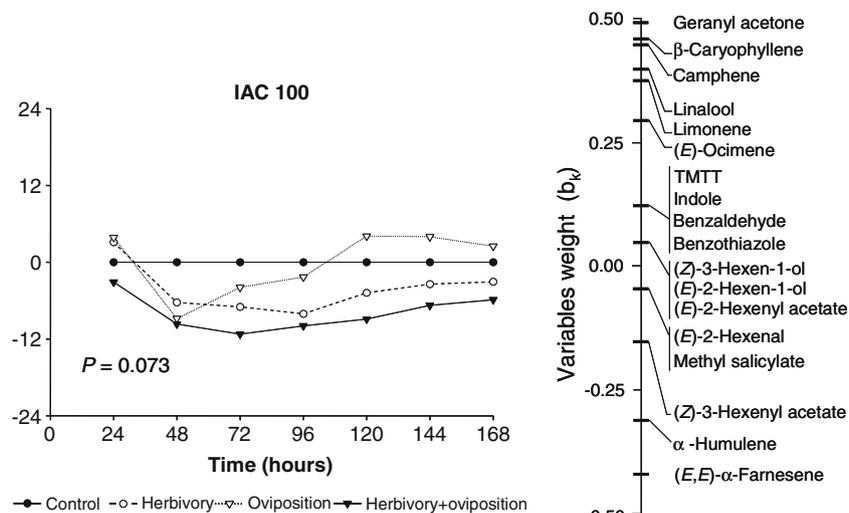
Fig. 7 PRC diagram and variables weights (b_k) based on volatile blends released by soybean plants of the cultivar IAC 100, on seven sampling occasions. The *lines* represent the response pattern of soybean plants to different treatments in time. The *P*-value indicates significance of the PRC diagram over all sampling times based on *F*-type permutation test. The first PRC did not display a significant part of the treatment variance (Monte Carlo permutation test, $P>0.05$), indicating that no significant induction treatment effect on the volatile blend composition was detected. All compounds showed low values for the variables weights ($b_k<0.5$)
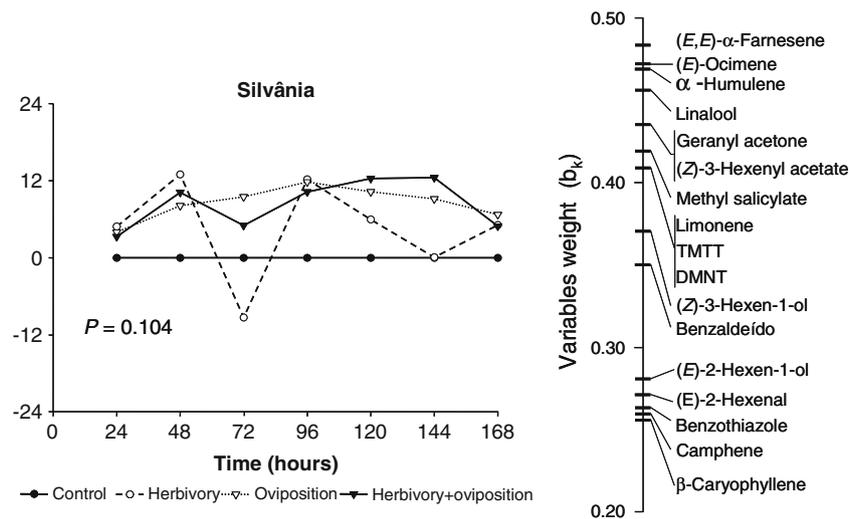
Fig. 8 PRC diagram and variables weights (b_k) based on volatile blends released by soybean plants of the cultivar Silvânia, on seven sampling occasions. The lines represent the response pattern of soybean plants to different treatments in time. The *P*-value indicates significance of the PRC diagram over all sampling times based on *F*-type permutation test. The first PRC did not display a significant part of the treatment variance (Monte Carlo permutation test, $P>0.05$), indicating that no significant induction treatment effect on the volatile blend composition was detected. All compounds showed low values for the variables weights ($b_k<0.5$)

volatile blends of these cultivars between undamaged, herbivory- and herbivory+oviposition-damaged soybean plants. Moraes et al. (2008) suggested that the egg parasitoid *T. podisi* was not attracted to the blend of volatiles produced by herbivory+oviposition and oviposition damaged soybean (cv BR-16), probably due the switching-off of jasmonate signaling caused by oviposition damage. In contrast, the results presented here suggest that oviposition did not switch off jasmonic acid-dependent plant defense because herbivory+oviposition-damaged soybean plants also attracted the natural enemy.

The lack of attraction of the egg parasitoid to oviposition-damaged plants could be explained if the response of the plant is not systemic. When the stink bug lays eggs on the leaves, there is a localized necrosis below and around the eggs (personal observation), which suggests a hypersensitive response (HR) by the plant, isolating the damaged area in a way similar to plant-pathogen responses (Walling, 2000; Kaloshian and Walling, 2005). What induces soybean plants to respond with necrosis to oviposition needs further study, but could be due to microorganisms or chemicals from the oviposition secretion that induce the HR. We are trying to elucidate this phenomenon by studying the chemistry of the saliva and the oviposition secretions.

The PRC analysis used in this work showed the main compounds induced by herbivore damage and the interaction between the damage time and the volatiles released by the soybean plant. The PRC analysis is a multivariate method that is commonly used to aid in impact assessments of pollutant stress on aquatic and terrestrial

biological communities in ecotoxicological studies (van den Brink and ter Braak, 1999; van den Brink et al., 2003), and application to other ecological aspects (Torres and Ruberson, 2005; Poelman et al., 2010) has proceeded rapidly. The great advantage of the PRC compared to other multivariate analysis is that it is possible to evaluate the variation of the compounds over time and the importance of each compound in a blend. Furthermore, there is no limitation on the number of variables and normality of the data is unnecessary.

The PRC results suggest that the increase of (*E,E*)-α-farnesene, methyl salicylate, (*Z*)-3-hexenyl acetate, and (*E*)-2-octen-1-ol in the total blend have an effect on parasitoid behavior, but the role of these compounds, individually, on the attraction of the egg parasitoid *T. podisi* needs to be evaluated. The attraction of parasitoids and predators to herbivore induced volatiles is often attributed to the relative contribution of each compound in the blend (D'Alessandro et al., 2006, 2009; Pareja et al., 2009), and not all herbivore-induced compounds are important for changing insect behavior. For example, two important compounds in the herbivore-induced volatile blend, indole in maize, *Zea mays*, and methyl salicylate in *Arabidopsis* sp, were not the key compounds responsible for the attraction of the parasitoids tested (D'Alessandro et al., 2006, 2009; Snoeren et al., 2010).

Telenomus podisi did not distinguish among the odors of damaged and undamaged plants for any treatment in cv Silvânia, probably because the volatile compounds released by undamaged and damaged Silvânia soybean are too similar for a discrimination. Vet et al. (1998) suggested that

an insect does not discriminate between odor sources because it is not able to do so, or because the odor is not in the right conditions for the discrimination, i.e., the compounds released by the plant are not in the correct concentration or proportion to be perceived by the natural enemies as a cue indicating the presence of the host. The egg parasitoid *T. podisi* discriminated between volatiles released from differently treated cv Dowling and IAC100, respectively. However, no preference for odor of any treatment of the susceptible Silvânia cultivar was detected; thus, we suggest that the susceptible cultivar was not induced by herbivore damage.

Herbivory-induced plant volatiles are important cues for host foraging behavior of parasitoids. Therefore, maintaining and even enhancing the attractiveness of crop plants to natural enemies should be considered an important trait when breeding new crop varieties. However, these possibilities may sometimes be constrained by the effect of these changes on the attractiveness of the plant to herbivorous pests, as volatiles that attract more natural enemies could also attract more herbivores (Cortesero et al., 2000). Thaler (1999) mentioned that it is advantageous for herbivores to avoid laying eggs on induced plants because such plants are likely to host other herbivores, which are potential competitors for second offspring herbivores, and can also attract a population of natural enemies. The results obtained in this work showed that *E. heros* does not use herbivore-induced volatiles as cues to distinguish between damaged and undamaged plants, or to distinguish between cultivars, but *E. heros* females responded preferentially to undamaged soybean (χ^2, $P=0.002$) compared to air, suggesting that *E. heros* uses the volatiles from soybean plants to locate its host-plant (results not shown).

The cv Dowling and IAC100 are known to have in their reproductive stage an antixenotic and antibiotic effect against aphids and stink bugs, respectively (Carrão-Panizzi and Kitamura, 1995; Li et al., 2004, 2008). Recently, field experiments showed that population levels of stink bugs colonizing cv Dowling and cv IAC100 were lower than on cv Silvânia suggesting a differential resistance/susceptibility (Laumann et al., 2008). Different studies suggested that the resistance during the reproductive stage of soybean could be related to the amount of isoflavonoids (Rao et al., 1990; Hoffmann-Campo et al., 2001; Piubelli et al., 2003, 2005). Experiments are needed to determine the amount of isoflavonoids during the different physiological stages of soybean plants to evaluate if there is a relation between direct and indirect defense and the quantities of volatile organic compounds.

In summary, the results suggest that the herbivorous stink bug *E. heros* induces the indirect defense of the resistant soybean cultivars Dowling and IAC100, and that the egg parasitoid *T. podisi* is attracted to the volatiles released by

herbivory- and herbivory+oviposition-damaged plants of these cultivars. In olfactometer bioassays, *E. heros* did not distinguish between damaged and undamaged soybean nor between resistant and susceptible cultivars. In addition, the resistant cultivar Dowling and IAC100 showed consistency in the main volatile compounds induced: (*E,E*)-α-farnesene, methyl salicylate, (*Z*)-3-hexenyl acetate. Further laboratory studies are necessary to investigate the importance of these compounds for attraction of the egg parasitoid *T. podisi*. Once understood, these volatile compounds could help to develop new strategies for control of *E. heros* using natural enemies in soybean crops.

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