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Interaction of *Anthonomus grandis* and cotton genotypes: biological and behavioral responses

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Abstract

The boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), is a key pest of cotton, *Gossypium hirsutum* L. (Malvaceae). Knowledge about boll weevil feeding and oviposition behavior and its response to plant volatiles can underpin our understanding of host plant resistance, and contribute to improved monitoring and mass capture of this pest. Boll weevil oviposition preference and immature development in four cotton genotypes (CNPA TB90, TB85, TB15, and BRS Rubi) were investigated in the laboratory and greenhouse. Volatile organic compounds (VOCs) produced by TB90 and Rubi genotypes were obtained from herbivore-damaged and undamaged control plants at two phenological stages – vegetative (prior to squaring) and reproductive (during squaring) – and four collection times – 24, 48, 72, and 96 h following herbivore damage. The boll weevil exhibited similar feeding and oviposition behavior across the four tested cotton genotypes. The chemical profiles of herbivore-damaged plants of both genotypes across the two phenological stages were qualitatively similar, but differed in the amount of volatiles produced. Boll weevil response to VOC extracts was studied using a Y-tube olfactometer. The boll weevil exhibited similar feeding and oviposition behavior at the four tested cotton genotypes, although delayed development and production of smaller adults was found when fed TB85. The chemical profile of herbivore-damaged plants of both genotypes at the two phenological stages and time periods (24–96 h) was similar qualitatively, with 30 identified compounds, but differed in the amount of volatiles produced. Additionally, boll weevil olfactory response was positive to herbivory-induced volatiles. The results help to understand the interaction between *A. grandis* and cotton plants, and why it is difficult to obtain cotton genotypes possessing constitutive resistance to this pest.

Introduction

Plant responses to herbivory include reduced nutritional quality and induced defensive compounds such as proteinase inhibitors and digestibility reducers. Secondary plant compounds, some of which can be herbivore-induced, often toxic to non-adapted herbivores (Karban & Baldwin, 1997; Walling, 2000; Schoonhoven et al., 2005; Arimura et al., 2009), may be used by adapted specialist herbivores for host plant recognition (Bolder et al., 1997; Addesso & McAuslane, 2009; Szendrei et al., 2009; Addesso et al., 2011; Magalhães et al., 2012). For instance, the cotton plant is rich in gossypol, which is associated with plant defense to herbivory; however, specialist herbivores such as the noctuid *Alabama argilacea* (Hübner) are adapted to this compound (Santos & Boiça Jr, 2002). Meanwhile, non-specialist herbivores such as the noctuid *Helicoverpa zea* (Boddie) are negatively affected by gossypol (Stipanovic et al., 2006). Plants also produce volatile organic compounds (VOCs) locally and systemically after being damaged by herbivore feeding or oviposition (Hare, 2007; Dicke & Baldwin, 2010; Gullan & Cranston, 2010). These VOCs can serve herbivores either as attractants to

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find host plants, or as repellents that help them to avoid patches with competitors (conspecifics or not) or herbivore-damaged plants that may contain induced defensive compounds (Kalberer et al., 2001; Meiners et al., 2005; Schoonhoven et al., 2005; Mérey et al., 2013). In addition to producing herbivore-induced VOCs, some plants increase the production of secondary compounds some of which serve as feeding deterrents affecting insect development (Bernasconi et al., 1998).

The boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), is considered the major pest of cotton, *Gossypium hirsutum* L. (Malvaceae), in the Americas. It is able to disperse long distances searching for the host plant or refuge areas. Cotton fields are colonized by boll weevils predominantly during squaring-flowering stages (Neff & Vanderzant, 1963; Smith et al., 1965) and weevil oviposition induces abscission of these structures 5–8 days later (White & Rummel, 1978; Showler, 2008; Neves et al., 2013), resulting in direct yield loss. Several studies have shown that VOCs play important roles in the attraction of the weevils to cotton plants (White & Rummel, 1978; Dickens, 1984, 1989, 1990; Magalhães et al., 2012).

When male boll weevil start feeding on cotton squares, their pheromone production increases, intensifying the attraction and aggregation of more males and females in the area (White & Rummel, 1978; Leggett, 1986). In addition, boll weevil captures in commercial traps (using the sex pheromone 'grandlure') decline considerably during the squaring-flowering stage of cotton (Lloyd, 1986; Rummel & Curry, 1986; Neves, 2013) suggesting that host plant volatiles produced at reproductive stage may modulate boll weevil attraction to its pheromone. Through electroantennography and olfactometry techniques, Dickens (1984, 1989) demonstrated that the boll weevil is attracted to cotton green leaf volatiles. Olfactory receptor neurons of boll weevils also were found capable of sensing odors emanating from cotton plants (Dickens, 1990). Combining cotton volatiles such as β-caryophyllene, (*E*)-2-hexen-1-ol, and (*E*)-β-ocimene with 'grandlure' in traps resulted in far more weevils being captured compared to 'grandlure' alone (Dickens, 1990). These findings support the hypothesis that cotton herbivory-induced VOCs can alter boll weevil behavior, and that these induced VOCs could be used to develop improved strategies to monitor and control this pest in cotton areas. Therefore, it is of major importance to understand how the boll weevil responds biologically and behaviorally to newly developed cotton genotypes prior to commercial field release because these data could mean less plant infestation in the field in case of plant resistant varieties. In fact, it has been previously known that cotton varieties can differ in the quantity and sometimes in quality of VOCs emission and these changes may be due to herbivory and plant phenological stage (Loughrin et al., 1995; Magalhães et al., 2012). Also differences in cotton VOC emission could be a side effect of plant genetic engineering for insect resistance (e.g., Bt-cotton) (Moraes et al., 2011) or conventional plant breeding for agronomic purposes, such as higher yield, what can have a direct impact on host plant location by herbivores such as the boll weevil.

Even though insecticides are massively applied to control the boll weevil (Lima Junior et al., 2013), their efficacy is reduced because of the protection afforded to the developing boll weevil immature stages inside cotton squares and bolls. Therefore, due to the difficulty of control after weevil establishment in an area, an alternative approach could be to interfere, reduce, or avoid the process of host plant location and acceptance by initially colonizing weevils from refuge areas after reproductive diapause ceases. Toward this goal, over the years various cotton genotypes have been selected for resistance (antibiosis and non-preference) to the boll weevil and recently one preliminary study has suggested that the cotton genotypes CNPA TB90 and TB85 showed some resistance to the boll weevil (Beltrão et al., 2001), but it is not clear whether this is a case of plant resistance through antibiosis or non-preference.

By definition, plant resistance through antibiosis occurs when an insect herbivore feeds normally on the plant, and this plant causes an adverse effect on the insect biology, affecting its fitness directly or indirectly (Bueno et al., 2001). Some causes of plant antibiosis are toxins, growth and/or reproduction inhibitors, and lack of nutrients, especially the C:N ratio. Therefore, cotton genotypes resistant to boll weevil through antibiosis would prevent or impair weevil's development on the cotton plant. Other adverse effects of antibiosis on insects would be death of young stages, size and weight reduction, low female fecundity, and low survival (Painter, 1968).

On the other hand, plant resistance through non-preference means that an herbivore shows less preference for a specific plant variety in comparison to other varieties offered in the same conditions, for feeding, oviposition, or shelter (Bastos et al., 2008). Non-preference may be due to a series of plant traits (e.g., more trichomes, leaf thoughness, plant structure, changes in VOC emission, etc.) (Schoonhoven et al., 2005). Therefore, if this is the case for those cotton genotypes (TB90 and TB85), we would expect a reduction in the number of colonizing weevils on a resistant cotton plant. Additionally, other cotton genotypes have been recently developed for the semiarid region of Brazil according to agronomic characteristics, but there is no report of their impact on the boll weevil population.

Therefore, the objectives of our study were to investigate whether different cotton genotypes negatively affect boll weevil development and whether herbivore-induced VOCs emitted by two of the selected genotypes differed and whether these compounds could affect boll weevil attraction. For this the following hypotheses were proposed: (1) the cotton genotype TB90 adversely affects host plant preference and boll weevil development, (2) herbivore-induced VOCs released by cotton plants vary with genotypes and phenological stage, and (3) herbivore-induced VOCs produced by cotton genotypes interfere with the host-seeking weevils' ability to locate, recognize, and accept the cotton plant.

Materials and methods

Plants

Cotton plants of the genotypes CNPA TB90, TB85, TB15, and BRS Rubi were cultivated in a greenhouse of the Agronomy Department of the Universidade Federal Rural de Pernambuco (UFRPE), Recife, Pernambuco, Brazil. Cotton seeds of the CNPA cultivars TB90, TB85, and TB15 (white fiber) were obtained from Embrapa Algodão, Campina Grande, Paraíba, Brazil, whereas seeds of the cultivar Rubi (colored fiber) were obtained from plants cultivated at UFRPE. Previous studies have shown that the genotypes TB90 and TB85 were less attacked by the boll weevil, suggesting that those genotypes have some degree of plant resistance to this pest (Beltrão et al., 2001). Genotypes Rubi and TB15 have been heavily attacked by the boll weevil in the field allowing us to use them as a positive control.

Plants were cultivated in a small greenhouse (6 × 2 × 2 m) in circular microplots (100 cm diameter, 50 cm deep) containing soil up to 20 cm from the upper border, and received N:P:K fertilizer (Yara Brasil Fertilizantes, Porto Alegre, RS, Brazil). Plants formed an equilateral triangle with three plants of the same genotype in each microplot, separated by ca. 30 cm. Fifteen and 30 days after emergence, each plant received 50 ml of ammonium sulfate (10 g l^{-1}) (Fertine, Fertilizantes do Nordeste, Recife, PE, Brazil).

Insects

Boll weevil adults used in experiments were originated either from infested cotton plants (CNPA 7H) cultivated at UFRPE campus or from a colony maintained on artificial diet at Embrapa Genetic Research and Biotechnology. Following Schmidt et al. (2001), the artificial diet used to rear the boll weevil was composed of a mixture of agar, beer yeast, wheat germ, soy protein, glucose, ascorbic and sorbic acid, Nipagin, flour from embryo of cottonseed (Pharmamedia®, Traders Protein, Lubbock, TX, USA), Wesson salt mixture, Vanderzant's vitamin, and water. Insects reared on artificial diet were used only to cause herbivory on cotton plants to be aerated at Embrapa Genetic Research and Biotechnology. In contrast, only boll weevil adults originating from cotton plants were used in biological and behavioral bioassays. Weekly, infested cotton squares and bolls were collected, placed in 500-ml plastic pots, and monitored for boll weevil emergence. Males and females were separated after emergence based on morphology differences of the abdomen tip (Sappington & Spurgeon, 2000). These isolated adults were fed cotton cotyledon leaves for 5 days, and then placed together in Plexiglas cages (45 × 45 × 30 cm) for mating. At the onset of mating, each couple was transferred to a Petri dish (3 cm diameter) containing cotton cotyledon leaves for 72 h. After this period, mated females were collected and used in further experiments.

Development of *Anthonomus grandis*

Cotton plants of the genotypes CNPA TB90, TB85, TB15, and BRS Rubi cultivated in the greenhouse and in the squaring/flowering stage were confined in cages as previously described, except that this time only one plant of each genotype was cultivated per microplot and caged. Two mated 9-day-old boll weevil females were released in each cage, and after 3 days the females were recovered from the cages (n = 12 for each cotton genotype). Punctured squares (oviposition sign) were marked with nail polish. In cotton, up to 60% abscission of reproductive structures is considered normal and this phenomenon is regulated by a balance between the amount of sugars and ethylene in plant tissue (Oosterhuis, 1992). In addition, attacked cotton squares are abscised from 5 to 8 days after boll weevil oviposition (Showler & Cantú, 2005), and were collected daily. Average boll weevil developmental time was considered as the time from squares attacked and fallen from plant until adult emergence, that is, development time in the greenhouse (from plant infestation with females to square abscission) and in the laboratory (from collection of fallen squares to adult emergence), as well as the total time from plant infestation to adult emergence.

Collected fallen squares were weighed using an analytical balance (precision 0.001 g, Model FA-2104N; Bioprecisa, São Paulo, SP, Brazil), and kept individually in 80-ml plastic containers until adult emergence, maintained at 25 ± 1 °C, 65% r.h., and L13:D11 photoperiod. Upon emergence, males and females were separated and weighed before feeding.

Feeding and oviposition preference of *Anthonomus grandis*

For these experiments we used only two cotton genotypes, one supposedly resistant to the boll weevil (CNPA TB90) and one susceptible (BRS Rubi). Choice trials were carried out in the Insect Behavior Laboratory at UFRPE, under conditions of 25 ± 1 °C, 67-75% r.h., and L13: D11 photoperiod. Additional choice trials were conducted in the greenhouse in which environmental conditions were monitored by Datalogger HOBO® (Onset Computer, Bourne, MA, USA) set up to register at 30-min intervals. The average temperature (± SD) was 27.2 ± 5.1 °C, r.h. was 76.7 ± 14.9%, and the natural photoperiod was ca. 12.8 h of light.

In the laboratory, choice and non-choice control trials were conducted using transparent plastic containers (500 ml) (Prafesta®, Mairipora, SP, Brazil) with lids containing an opening covered by a piece of 'voile' fabric for aeration. These containers constituted the choice arenas where squares were offered to boll weevil adults for oviposition. In each arena, water was added on the bottom, with a tightly fitted styrofoam disc (10 × 1 cm) above. Each disc had two small holes that were used to hold the petioles of the cotton squares in contact with the water below. Thus, the squares remained turgid throughout the 48-h experiment.

The experiment had three treatments consisting of choice and non-choice trials, with 20 replications each. Two treatments were non-choice trials (TB90 vs. TB90 and Rubi vs. Rubi), and the third was the choice trial (TB90 vs. Rubi). One 10-day-old female boll weevil was released in the center of each arena. Arenas were checked at 1, 6, 24, and 48 h after releasing the females, and the number of feeding and oviposition holes in the cotton squares was recorded, as well as the weevil's location.

In the greenhouse experiment, conducted separately, cotton plants of the genotypes TB90 and Rubi were cultivated as previously described, using two plants per microplot. The treatments were composed of two plants of the same genotype (TB90 vs. TB90 and Rubi vs. Rubi) as non-choice trials, and one plant for each of the two genotypes (TB90 vs. Rubi) as the choice trials, with eight replications each. Plant location in the microplots was randomly assigned for each replication within a treatment.

When plants were 46 days old, each microplot containing the plants was completely covered with a cylindrical cage (1.4 × 1.0 m) of aphid-proof screen. Cages had a lateral opening closed with Velcro™ to allow plant inspection. The experiments were run when the plants were 50–60 days old with high production of squares and development of first bolls (Ritchie et al., 2007). Then, two 10-day-old mated boll weevil females were released inside the cages. After 3 days exposure to the weevils the plants were carefully inspected, recording the presence or absence of the boll weevils on each plant, the total number of squares per plant, the number of punctured squares (feeding damage and oviposition), and the total number of fruiting structures available per plant.

Volatile collections

Plant VOC collection was conducted at the Semiochemical Laboratory of Embrapa Genetic Research and Biotechnology. Cotton plants of the genotypes CNPA TB90 and BRS Rubi were cultivated in the greenhouse under natural conditions of temperature (range 13–30 °C) and photoperiod of ca. 11 h of light at Embrapa using 5-l plastic pots containing a mixture of soil and humus (2:1). Fertilizer [5 g 4:30:16 (N:P:K) per plant] was applied at planting, and 4 ml of ammonium sulfate solution (20%) was applied 30 days after germination. Plants remained in the greenhouse until their use for headspace collection.

Cotton plants used for collection of volatiles were taken from the greenhouse to the laboratory 1 day before the collection. After watering, the pots and soil were covered with aluminum foil, to minimize contamination with VOCs from these sources. Volatiles were collected from plants at two stages: vegetative stage when plants had a minimum of six expanded leaves (i.e., prior to square production), and reproductive stage when plants had at least one developed square.

Boll weevils used to cause herbivory on plants submitted to VOC collection originated from a laboratory colony reared on artificial diet following Magalhães et al. (2012). In addition, to encourage immediate feeding after being placed on plants, the 10-day-old adult boll weevil females were starved for 24 h prior to experiments. Weevil damage to plants includes small punctures on leaves and plant terminals, which do not allow precise calculations of leaf area consumed for later correlation with cotton volatile production.

Plant VOC collection was conducted in a 12-l cylindrical glass chamber (50 × 17.5 cm), in a room with controlled environmental conditions (25 ± 1 °C and of 65 ± 10% r.h.). Plants were placed individually in each glass chamber and plants subjected to herbivory received two boll weevil females. Each chamber had a small hole (1 cm diameter) on the top portion, into which glass tubes containing the adsorbent polymer could be inserted, one tube per chamber. Before VOC collection, the glass tubes (15 cm × 5 mm) containing the adsorbent Porapak-Q (80-100 mesh; Supelco, Bellefonte, PA, USA) were preconditioned for 12 h at 250 °C in a constant nitrogen flux. Each glass tube was connected via PTFE tubing to a vacuum pump at a flow of 0.6 l per min; the air entrance was connected to a flow of charcoal-filtered air (0.8 l per

min), therefore ensuring that purified air entered the system and creating a positive pressure, 'push-pull' system.

The experiment was conducted using a complete randomized factorial design (2 × 2 × 2), totaling six treatments with 12 replications each. The treatments corresponded to two genotypes (TB90 and Rubi), two phenological stages (vegetative and reproductive), and either undamaged or herbivory-damaged. Twelve independent chambers were run simultaneously, enabling simultaneous VOC collections for 24 h over four consecutive days from all treatments.

The trapped VOCs were eluted from the adsorbent with 2 ml of *n*-hexane, concentrated to 100 µl by a nitrogen flow, transferred to 2-ml glass vials, and stored at −20 °C. Half of each extract was used in subsequent behavioral bioassays with boll weevil, and the other 50 µl was used for chemical analyses [gas chromatography (GC) with flame ionization detection (FID) and GC/mass spectrometry (MS)].

Chemical analysis

The analyses of the compounds in the plant extracts was conducted with 1 µl of 16-hexadecanolide added as an internal standard (IS), at final concentration of 0.002 mg ml^{-1}. Extracts of plant VOCs were analyzed by GC [Shimadzu 17A, DB-5 column, 0.25 mm inner diameter (ID) × 30 m, 0.25 µm film; J&W Scientific, Folsom, CA, USA], with the column oven programmed at 50 °C for 2 min, then to 180 °C at 5 °C per min, held for 0.1 min, followed by an increase of 10 °C per min to 250 °C (held for 20 min). The FID was at 270 °C. Aliquots of 1 µl of each sample were injected using splitless mode, with helium as carrier gas. Data were collected using Shimadzu Class GC software and were analyzed using Excel Software (Microsoft, 2007). Analysis of the compounds from the plant extracts was conducted with 1 µl of 16-hexadecanolide added as an internal standard (IS), at a final concentration of 0.002 mg ml^{-1}. Then, the quantification was done comparing the area of the IS with the areas of all compounds in the chromatogram profile. The response factor of the detector was considered equal to one.

After sample injections in the GC-FID and analyses of the chromatograms, the most representative four samples were selected for injection into the GC-MS for qualitative analysis. The chosen samples were injected on an Agilent 5975C quadruple mass spectrometer equipped with a DB-5 column (30 m × 0.25 mm ID, 0.25 µm film; Supelco, Bellefonte, PA, USA) and a splitless injector, with helium as carrier gas. Ionization was done by electron impact (70 eV, source temperature 200 °C). Data were collected with ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Identifications were made by comparison of spectra with library databases (Software NIST-Wiley database, version 2.0, 2008, Agilent Technologies, Wilmington, DE, USA) or with published spectra and using retention index (published at Pherobase and NIST Chemistry Web Book websites) and confirmed using authentic standards when available.

The chemical compounds α-pinene (98%), camphene (75%), benzothiazole (96%), β-pinene (99%), ocimene (90%), γ-terpinene (97%), (*Z*)-3-hexenyl acetate (98%), limonene (97%), benzaldehyde, indole (98.5%), methyl salicylate (99%), α-copaene (90%), α-caryophyllene, alloaromadendrene (90%), and β-farnesene were purchased from Sigma Aldrich (Steinheim, Germany). Myrcene (analytical standard) was obtained from Fluka (Buchs, Switzerland), linalool and β-caryophyllene from TCI-America (Portland, WA, USA), and geranylacetone and *cis*-jasmone from TCI-Japan (Tokyo, Japan). The compounds (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were provided by Dr. Michael Birkett (Rothamsted Research, Harpenden, UK). The hexane HPLC grade was obtained from Sigma Aldrich and redistilled before use.

Boll weevil response to cotton plant volatiles

Olfactory responses of boll weevil adults were investigated in the Insect Behavior Laboratory at UFRPE campus. The bioassays used boll weevil adults emerged from infested cotton squares and bolls of the cultivar NuOPAL RR® collected from a cotton field located in Surubim County, Pernambuco, Brazil. The behavioral responses of sexually mature virgin boll weevil males and females, 5–10 days old, were evaluated using a two-choice Y-tube olfactometer (Magalhães et al., 2012). Virgin adults were used because it was previously shown that *A. grandis* males are first attracted to cotton plants and after feeding on cotton squares begin to release aggregation pheromone, which in turn attracts more individuals to the area, when mating and oviposition occurs (Tumlinson et al., 1969; Dickens, 1989). Our preliminary tests showed that boll weevil adults did not respond preferentially to volatiles of either cotton genotype (BRS Rubi and CNPA TB90) in the vegetative stage vs. the control (clean air) (data not shown). Therefore, we continued our experiments using only VOCs released by plants in the reproductive stage. Thus, insects in the bioassay were exposed to cotton VOCs extracted for 24 or 96 h from the plant genotypes TB90 and Rubi in the reproductive stage and exposed or not to herbivory.

For the bioassays, an aliquot of 15 µl of the plant extract was micropipetted onto filter paper (1.5 cm^2, 89 g m^{-2}, 0.18–0.19 mm thick, mean number of pores = 14.7 μm^{-1}, volume of pores = 72%). The solvent was

allowed to evaporate for 1 min at room temperature prior to testing. Filter papers containing the extracts were placed inside glass syringes (10 ml), which were connected to the arms of the olfactometer by PTFE tubing. Charcoal-filtered and humidified air was pushed through the system by an aquarium air pump (Aleas® – AP9802, Chaozhou, Guangdong, China) regulated at a flow rate of 0.1 l per min by flowmeters (KI®, Hatfield, PA, USA) positioned at each arm of the olfactometer, and a suction pump (Bioblock Scientific®, Illkirch Cedex, France) was used to pull the air from the system at a rate of 0.2 l per min, also regulated by a flowmeter.

Prior to the bioassays, adult boll weevils were starved during 24 h to induce the host plant searching behavior. One starved boll weevil adult, male or female, was introduced in the stem arm of the olfactometer and the response to odors offered in each arm of the olfactometer was observed for 10 min. When the boll weevil passed the bifurcation point of the Y-tube into one arm of the olfactometer and traveled for at least 3 cm into its length and stayed there for at least 20 s, it was considered to have made a first choice. The total time that a boll weevil spent in each Y-tube arm was also recorded. Each weevil was tested only once, and the filter papers impregnated with the plant VOC extracts were changed after every five replications and included rotating the position of treatments offered to avoid any bias in the insect response. The Y-tube apparatus was cleaned with soap and water and dried after every 20 trials. All experiments were done during the photophase, between 09:00 and 17:00 hours. A total of 40 replicates were run for each pair of treatments using 20 males and 20 females. Odor sources offered were herbivore-damaged TB90/Rubi vs. hexane control; undamaged TB90/Rubi vs. hexane; undamaged/herbivore-damaged Rubi vs. undamaged/herbivore-damaged TB90; herbivore-damaged Rubi vs. undamaged Rubi; herbivore-damaged TB90 vs. undamaged TB90.

Statistical analysis

The total numbers of insects observed per plant and the numbers of squares with oviposition and feeding punctures in the first laboratory experiment regarding oviposition preference were submitted to the non-parametric Proc FREQ of SAS (SAS Institute, 2001) followed by a χ^2 test ($\alpha = 0.05$), with the null hypothesis that no difference in host plant preference exists between the two cotton genotypes (1:1 ratio). Host plant preference trials conducted in the greenhouse were analyzed by paired-t tests using Proc TTEST (SAS), comparing the plant genotypes chosen, the average numbers of attacked squares, and the numbers of flowers and bolls per plant. Weight data of fallen squares, boll weevil developmental time, and adult (male and female) weights were subjected to a Kolmogorov-Smirnov normality test and a variance homogeneity test (Bartlett's test) using Proc UNIVARIATE (SAS). The developmental time and adult weights were $\sqrt{(x + 0.5)}$ transformed, to meet the assumptions of ANOVA; untransformed means are presented in the results. Data were submitted to ANOVA, with cotton genotype and boll weevil sex as main factors, using Proc GLM (SAS). This was followed by Tukey's honestly significant difference (HSD) test for comparisons of means ($\alpha = 0.05$; SAS). In addition, the relationships between boll weevil developmental time and adult fresh body weight as a function of square size were subjected to regression analysis using Proc REG (SAS). The best fit model was selected based on significance levels of the parameters, a high adjusted coefficient of determination, and the F-value.

To analyze the total amount of VOCs released by cotton plants under boll weevil herbivory during the two phenological stages, data were subjected to a normality test (Kolmogorov-Smirnov) and a variance homogeneity test (Bartlett), and log $(x + 1)$ transformed to meet the assumptions of ANOVA. In addition, because volatile collections were taken from the same cotton plants at different times (24, 48, 72, 96 h) a repeated measures General Linear Model (Proc GLM of SAS) was used following a factorial design with three main factors: cotton genotype (BRS Rubi and CNPA TB90), plant phenological stage (vegetative and reproductive), status of herbivory (undamaged and herbivore-damaged plant) with time collection as a blocking factor to avoid pseudoreplication. When the analyses showed significant effects of treatments, the average of VOCs was compared between cotton genotypes, plant phenological stage, and status of herbivory by Tukey's HSD ($\alpha = 0.05$; SAS). The change in chemical profile of herbivore-damaged and undamaged cotton plants over time was assessed using Principal Response Curves (PRC) analysis (van den Brink & ter Braak, 1999; Moser et al., 2007; Michereff et al., 2011). The PRC analysis was applied separately for each cotton genotype and phenological stage. In each set of analyses, the significance was determined by a Monte Carlo permutation test (number of permutations = 999). This analysis was performed using the statistical program R 2.14.0 (R Development Core Team, 2009).

In the olfactometer bioassays, data of first choice were submitted to the non-parametric Proc FREQ (SAS) followed by a χ^2 test ($\alpha = 0.05$), with the null hypothesis that no difference exists between the two odor sources offered (1:1 ratio). Residence time of the weevil in each arm of the olfactometer was analyzed by paired-t test using Proc TTEST (SAS).

Results

Development of *Anthonomus grandis*

Boll weevils exhibited similar developmental times ($F_{1,198}$ = 0.43, P = 0.51) and adult fresh body weight ($F_{1,198}$ = 0.64, P = 0.43) considering all insects (males and females) emerged. Therefore, subsequent results are presented only as a function of cotton genotypes. Development times in the greenhouse (from plant infestation to square abscission; $F_{3,202}$ = 9.20, P<0.0001) and in the laboratory (from collection of fallen squares to adult emergence; $F_{3,180}$ = 10.08, P<0.0001), as well as the total time from plant infestation to adult emergence ($F_{3,180}$ = 12.20, P<0.0001) and adult fresh body weight ($F_{3,180}$ = 3.99, P = 0.022) all differed as function of cotton genotypes. Weevil viability was similar among cotton genotypes and varied from 66.0 to 81.7% of emergence. In addition, the developmental time in the genotype CNPA TB85 averaged a day longer, followed by the genotype BRS Rubi (Table 1). The developmental time monitored in the laboratory correlated significantly with the size of the cotton squares in TB15 and TB85 (Figure 1).

Even though the developmental time of the weevil was longer in TB85, resulting adults were smaller than those that emerged from TB90 (Table 1). Moreover, the larger squares produced by TB85 did not result in larger adults (Table 1), although larger cotton squares raised larger boll weevils (Figure 1).

Feeding and oviposition preference of *Anthonomus grandis*

Boll weevils did not show consistent preference between tested cotton genotypes CNPA TB90 and BRS Rubi in the laboratory. The results varied depending on when the samples were collected, with higher numbers of boll weevil females on squares of Rubi (χ^2 = 4.04, d.f. = 1, P = 0.044) after 1 h exposure, and the opposite after 48 h exposure with more boll weevil females on TB90 (χ^2 = 6.42, d.f. = 1, P = 0.011). At the 6- and 24-h sample times the number of weevils per square did not differ between genotypes (6 h: χ^2 = 1.22, d.f. = 1, P = 0.27; 24 h: χ^2 = 3.43, d.f. = 1, P = 0.064). In addition, there was no significant difference in the boll weevil preference in non-choice control trials (TB90 vs. TB90, Rubi vs. Rubi). The number of punctures found in the cotton squares also did not differ in choice and non-choice trials, varying from one to two feeding punctures per square, regardless of trial or genotype.

In the greenhouse experiment oviposition preferences between TB90 and Rubi were similar (t = 0.09, d.f. = 1, P = 0.93). The mean (± SEM) number of attacked squares was 12.3 ± 1.82 on Rubi and 13.6 ± 2.21 on TB90 (t = 0.38, d.f. = 1, P = 0.71). The availability of squares and flowers was also similar between the genotypes with 41.8 ± 4.20 and 36.8 ± 4.52 squares and 4.9 ± 0.97 and 5.3 ± 1.16 flowers, respectively, on Rubi and TB90. As in the laboratory, there was no significant difference in the boll weevil preference in non-choice control trials (TB90 vs. TB90, Rubi vs. Rubi) in the greenhouse.

Chemical analysis

Thirty VOCs released by undamaged or herbivore-damaged plants for both genotypes BRS Rubi and CNPA TB90 were identified in the vegetative and reproductive stages (Table 2). In addition, analyses of selected extracts of VOC from cotton plants revealed no qualitative differences among treatments. Compounds identified by GC-MS included α-pinene (RI = 941), camphene (RI = 959), benzaldehyde (RI = 966), β-pinene (RI = 982), myrcene (RI = 991), (*Z*)-3-hexenyl acetate (RI = 1004), (*E*)-2-hexenyl acetate (RI = 1015), 1-decyne (RI = 1024), 2-ethylhexan-1-ol (RI = 1029), limonene (RI = 1035), (*E*)-β-ocimene (RI = 1050), γ-terpinene (RI = 1063), linalool (RI = 1098), (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (RI = 1115), (*E*)-2-hexenyl butyrate (RI = 1144), methyl salicylate (RI = 1194), benzothiazole (RI = 1229), indole (RI = 1293), α-copaene (RI = 1380),

Table 1 Mean (± SEM) developmental times and adult fresh body weight of *Anthonomus grandis* fed on the four cotton genotypes and weight of cotton squares

Genotype	Duration (days)[1] (infestation-abscission)	Duration (days)[2] (abscission-emergence)	Duration (days) (infestation-emergence)	Adult fresh body weight (mg)	Weight of squares (mg)
BRS Rubi	8.6 ± 0.18a	12.2 ± 0.18b	21.1 ± 0.51a	8.0 ± 0.43ab	457.7 ± 35.99ab
CNPA TB15	7.1 ± 0.29b	12.1 ± 0.12b	18.8 ± 0.26b	7.9 ± 0.36b	431.2 ± 21.29b
CNPA TB85	8.6 ± 0.26a	13.3 ± 0.22a	21.5 ± 0.30a	7.6 ± 0.37b	577.8 ± 48.25a
CNPA TB90	7.0 ± 0.30b	12.2 ± 0.13b	18.9 ± 0.26b	9.6 ± 0.54a	440.9 ± 27.86b

Means within a column followed by the same letter are not significantly different (Tukey HSD test: P>0.05).

[1]Developmental times were measured from the time of plant infestation with boll weevils to cotton square abscission from the plant in the greenhouse.

[2]Developmental times were measured from the time of cotton square abscission from the plant to adult emergence in the laboratory.

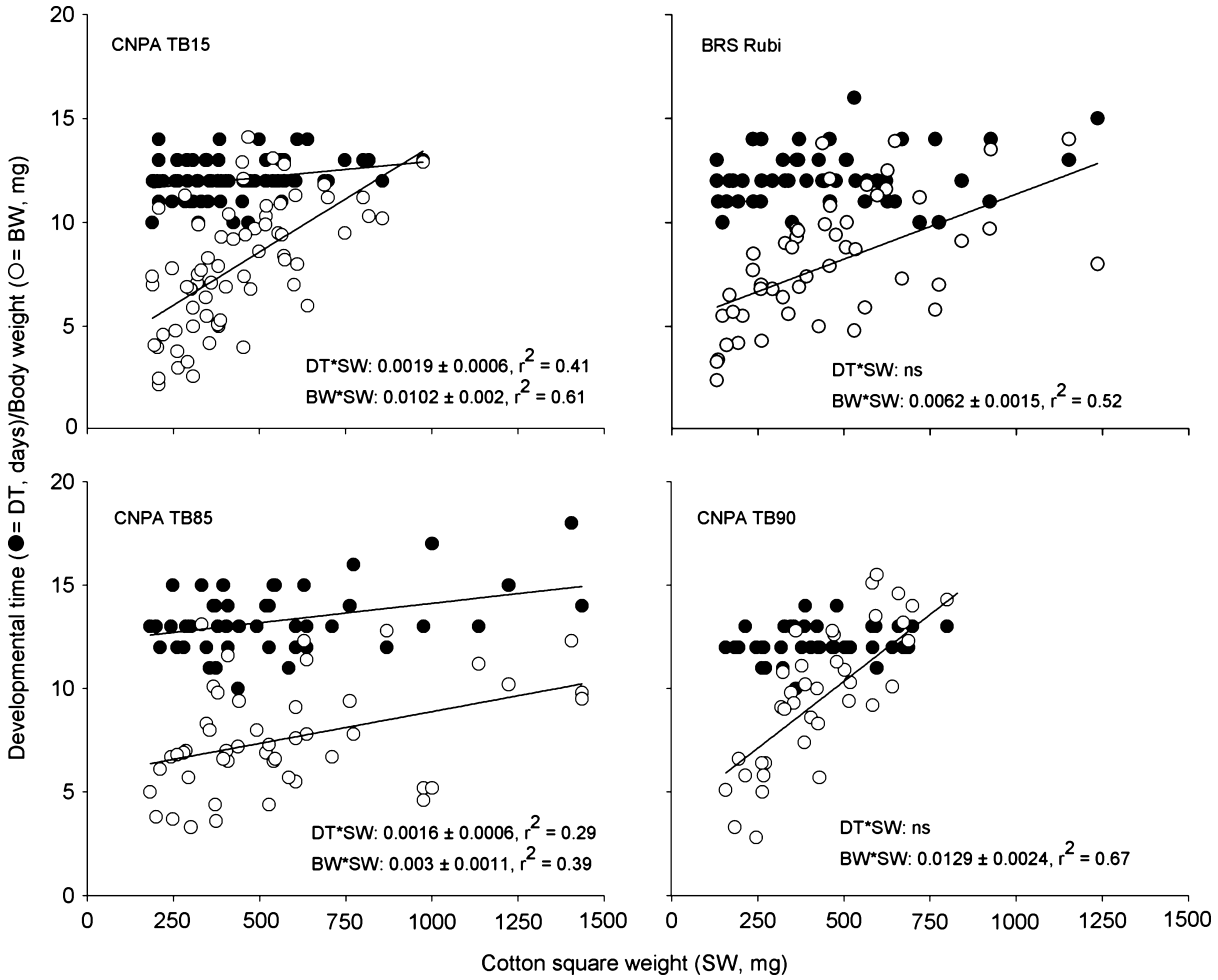

Figure 1 Developmental time (DT) (days) of *Anthonomus grandis* raised in cotton squares, and adult fresh body weight (BW) (mg) of weevils as function of cotton square weight (SW) (mg) for each cotton genotype used. Mean (± SEM) values given in the panels represent the slope (top) and coefficient of determination (bottom) for the respective linear regressions; ns, slope not significantly different from 0.

cis-jasmone (RI = 1394), β-caryophyllene (RI = 1425), guaiene (RI = 1437), geranylacetone (RI = 1449), β-farnesene (RI = 1454), α-caryophyllene (RI = 1461), alloaromadendrene (RI = 1465), germacrene D (RI = 1483), (*E*,*E*)-α-farnesene (RI = 1505), nerolidol (RI = 1566), and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (RI = 1575). From the compounds identified, the terpenes (sesquiterpenes and monoterpenes) were the most abundant, followed by esters and alcohols (Table 2).

Regardless of herbivory status (damaged or undamaged plants) and phenological stage, Rubi released more (2.4×) VOCs compared to TB90 (repeated measures ANOVA: $F_{1,81} = 45.24$, $P<0.0001$; Figure 2A). Furthermore, damaged plants of all genotypes released more volatiles than undamaged plants ($F_{1,81} = 15.36$, $P = 0.0002$; Figure 2B).

The amount of VOCs released by cotton genotypes differed between the sampling times comparing the genotypes, herbivory status, and phenological stage (Wilks' Lambda = 20.09, 6.47, and 2.74, respectively; $P<0.0001$, 0.0006, and 0.049, all d.f. = 3.79; Figure 3). Moreover, at all sampling times the greatest amount of VOCs released occurred in the reproductive stage of the plants ($F_{1,81} = 18.98$, $P<0.0001$), except for the 48-h sample in which there was no difference in the amount of VOCs released (Figure 3B). On average, 1.6× more VOCs were released in the reproductive stage than in the vegetative stage of plants (mean ± SEM = 147.3 ± 10.10 vs. 89.8 ± 7.22 ng per 24 h). In addition, 1.6× more VOCs were released when plants were damaged by herbivores, except for the 72-h sample (Figure 3C).

Table 2 Mean (± SEM) amount of volatile organic compounds (ng per 24 h) collected from 24 to 96 h from cotton genotypes CNPA TB90 and BRS Rubi, damaged by *Anthonomus grandis* (HD) or undamaged control (UD) plants, in the vegetative and reproductive stages

	BRS Rubi				CNPA TB90			
	Vegetative		Reproductive		Vegetative		Reproductive	
	UD	HD	UD	HD	UD	HD	UD	HD
Monoterpenes								
Camphene	162.2 ± 31.70b[1]	871.6 ± 108.24a	914.2 ± 221.95a	1012.0 ± 338.58a	33.4 ± 1.31c	152.7 ± 13.47b	97.4 ± 13.36bc	1151.7 ± 632.56a
α-Pinene	5.74 ± 1.31cd	15.7 ± 1.93abc	15.2 ± 2.60abcd	20.0 ± 3.79ab	5.1 ± 0.68d	7.5 ± 2.7cd	7.7 ± 1.13bcd	29.9 ± 14.28a
β-Pinene	36.6 ± 6.95c	188.5 ± 25.48a	173.5 ± 48.32ab	254.3 ± 41.25a	13.7 ± 1.02d	68.5 ± 6.93bc	40.2 ± 5.70c	347.3 ± 133.84a
Myrcene	115.8 ± 29.33d	501.4 ± 53.35ab	399.2 ± 108.25abc	587.9 ± 8.35a	38.9 ± 6.77e	266.5 ± 40.82bc	193.6 ± 14.43dc	765.7 ± 102.11a
Limonene	20.7 ± 2.02b	66.8 ± 9.27a	80.3 ± 19.31a	110.2 ± 16.03a	9.1 ± 0.82b	22.0 ± 2.14b	21.3 ± 2.69b	111.0 ± 50.82a
E-β-Ocimene	11.4 ± 1.17bc	21.1 ± 3.71ab	17.2 ± 1.63ab	28.8 ± 2.98a	7.8 ± 1.05c	19.4 ± 4.80ab	13.28 ± 1.41bc	28.1 ± 4.84a
γ-Terpinene	8.4 ± 0.38a	6.8 ± 0.89a	9.7 ± 1.90a	9.4 ± 1.02a	5.4 ± 0.56a	7.4 ± 1.65a	5.5 ± 1.71a	9.6 ± 3.66a
Linalool	28.9 ± 4.43bc	90.0 ± 17.05a	33.3 ± 5.86bc	87.6 ± 13.20a	21.2 ± 0.70bcd	52.6 ± 17.16ab	18.3 ± 3.34 cd	11.2 ± 2.06d
Sesquiterpenes								
Copaene	2.9 ± 0.46cde	11.0 ± 1.01ab	6.1 ± 0.41abc	14.5 ± 0.52a	2.4 ± 0.25de	6.6 ± 2.31bcd	2.1 ± 0.38e	6.5 ± 2.32bcd
cis-Jasmone	3.0 ± 0.09bc	11.8 ± 2.89a	6.7 ± 1.07abc	6.8 ± 0.63ab	2.1 ± 0.19c	6.1 ± 1.16abc	7.9 ± 3.14abc	2.8 ± 0.91bc
β-Caryophyllene	23.9 ± 2.87d	310.1 ± 36.32a	128.8 ± 17.83bc	241.3 ± 20.20ab	12.0 ± 2.51e	112.0 ± 12.99c	48.1 ± 4.63d	163.7 ± 35.38abc
Geranylacetone	17.5 ± 2.99a	14.2 ± 2.19a	15.3 ± 6.63a	19.8 ± 4.42a	12.5 ± 2.93a	14.2 ± 3.27a	12.7 ± 3.35a	11.4 ± 4.74a
β-Farnesene	7.1 ± 1.31ab	25.0 ± 7.42a	14.1 ± 0.10ab	17.1 ± 0.64a	5.7 ± 1.30ab	11.9 ± 5.51ab	5.9 ± 1.01ab	5.0 ± 1.23b
α-Caryophyllene	16.7 ± 1.10b	92.5 ± 11.16a	51.0 ± 6.79ab	80.0 ± 5.05ab	17.9 ± 3.32b	45.9 ± 5.19ab	8.8 ± 69.53ab	63.7 ± 13.32a
Alloaromadendrene	3.9 ± 1.80ab	3.7 ± 0.70ab	10.8 ± 2.28a	8.1 ± 1.61a	1.6 ± 0.30b	13.0 ± 8.02a	81.2 ± 2.61ab	4.2 ± 0.57ab
Germacrene D	6.5 ± 0.52ab	14.7 ± 3.45a	6.6 ± 0.49ab	8.4 ± 0.65ab	6.2 ± 0.55b	13 ± 2.00ab	6.4 ± 0.70b	13.0 ± 2.54ab
α-Farnesene	10.8 ± 1.34abc	10.7 ± 1.35abc	10.4 ± 1.97bc	18.8 ± 2.99ab	6.8 ± 0.52c	28.4 ± 9.40a	4.6 ± 0.57c	11.2 ± 3.79bc
Guaiene	2.4 ± 0.30ab	2.3 ± 0.37ab	2.5 ± 0.14ab	3.2 ± 0.33ab	2.5 ± 0.21ab	4.4 ± 1.19a	1.9 ± 0.22b	3.1 ± 0.74ab
Nerolidol	12.7 ± 2.57ab	10.0 ± 1.46ab	16.5 ± 3.20ab	20.4 ± 3.89a	7.3 ± 0.75b	7.5 ± 1.00b	99.4 ± 1.88ab	6.9 ± 0.89b
Esters								
Benzaldehyde	14.2 ± 1.45a	25.2 ± 6.94a	23.1 ± 4.39a	27.4 ± 2.66a	9.8 ± 0.53a	18.3 ± 4.85a	42.3 ± 22.95a	15.8 ± 2.3a
Methyl salicylate	141.5 ± 82.49ab	51.6 ± 23.00abc	104.0 ± 36.89abc	208.7 ± 69.04a	53.2 ± 25.94abc	34.2 ± 11.29abc	4.7 ± 0.77c	6.5 ± 1.45bc
Benzothiazole	5.9 ± 0.60a	213.9 ± 207.68a	8.9 ± 3.44a	39.5 ± 13.90a	7.6 ± 0.48a	34.4 ± 4.57a	8.1 ± 1.55a	12.0 ± 2.00a
Indole	8.7 ± 0.76a	29.9 ± 9.56a	7.2 ± 2.84a	11.3 ± 1.19a	7.7 ± 0.46a	9.0 ± 3.39a	44.5 ± 1.01a	4.7 ± 0.86a
Z-3-Hexenyl acetate	63.8 ± 19.15cd	1180.1 ± 304.64a	138.5 ± 60.52bc	252.0 ± 69.46b	23.4 ± 3.86d	269.6 ± 39.99b	55.7 ± 5.87cd	43.2 ± 13.80cd
E-2-Hexenyl acetate	3.2 ± 0.25a	8.9 ± 5.35a	3.4 ± 0.67a	5.3 ± 0.97a	3.1 ± 0.54a	3.9 ± 0.96a	7.1 ± 3.60a	3.4 ± 0.63a
E-2-Hexenyl butyrate	3.9 ± 1.13a	127.0 ± 122.09a	3.6 ± 1.28a	6.2 ± 3.19a	14.2 ± 7.52a	17.5 ± 3.31a	17.5 ± 0.52a	3.6 ± 2.00a
Homoterpenes								
DMNT[3]	571.6 ± 168.58abc	784.5 ± 214.06ab	1033.6 ± 281.73ab	1287.6 ± 388.01a	187.7 ± 21.00bc	281.9 ± 64.50abc	410.9 ± 91.26abc	156.3 ± 71.58c
TMTT[2]	1116.8 ± 379.43a	702.8 ± 262.65a	1654.3 ± 544.71a	1968.1 ± 649.12a	513.6 ± 35.42a	434.6 ± 78.14a	897.3 ± 304.46a	153.3 ± 55.68a
Alcohols								
1-Decyne	38.0 ± 5.54de	54.6 ± 13.27bcde	163.7 ± 11.88a	93.9 ± 7.86abcd	22.9 ± 2.85e	56.4 ± 22.7cde	106.1 ± 18.52abc	131.2 ± 18.25ab
Ethylhexanol	19.0 ± 2.12a	23.2 ± 8.54a	25.9 ± 8.25a	30.1 ± 8.68a	13.5 ± 1.34a	18.7 ± 4.25a	32.7 ± 13.31a	54.5 ± 22.17a

Means within a row followed by the same letter are not significantly different (Tukey HSD test: $P>0.05$).

[1](*E*)-4,8-dimethyl-1,3,7-nonatriene.

[2](*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

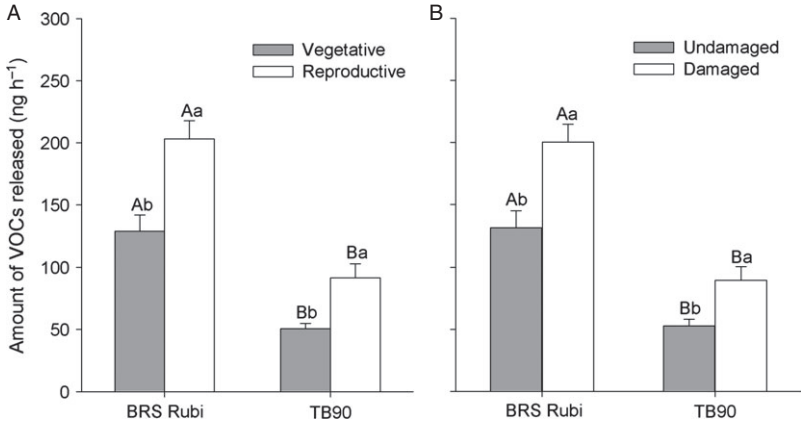

Figure 2 Mean (+ SEM) total production of volatile organic compounds (VOCs) (ng h^{-1}) of cotton genotypes BRS Rubi and CNPA TB90, as a function of (A) plant phenological stage and (B) herbivory status (damaged or undamaged). Bars capped with the same capital letter comparing phenological stage or herbivory status do not differ between cotton genotypes; bars capped with the same small letter do not differ comparing phenological stages or herbivory status within cotton genotype (Tukey HSD test: $P>0.05$).

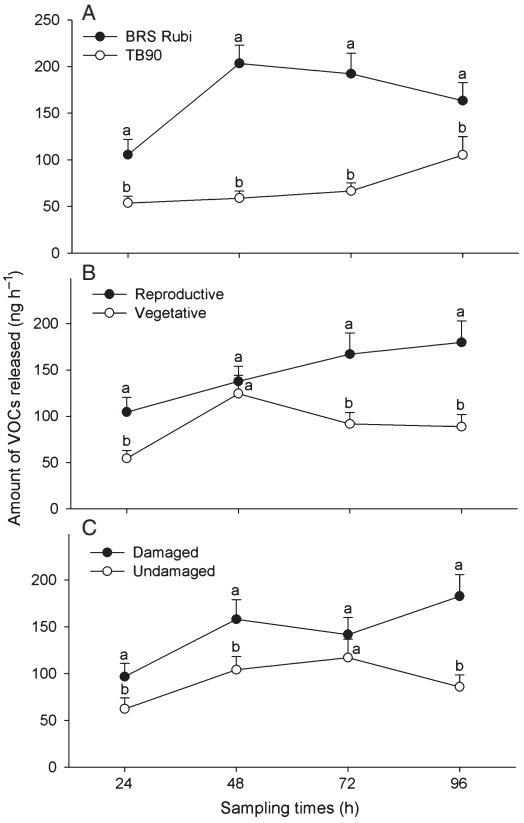

Figure 3 Mean (+ SEM) amount of volatile organic compounds (VOCs) (ng h^{-1}) (A) released from cotton genotypes, (B) plant phenological stage, and (C) herbivory status (damaged or undamaged) as function of sampling time. Means within a panel and within a sampling time capped with different letters are significantly different (Tukey HSD test: $P<0.05$).

PRC analysis evaluated differences in VOCs between herbivore-damaged vs. undamaged plants, between phenological stages, and it identified the main volatile compounds responsible. Cotton plants in the various treatments showed variability over time and among treatments. The importance of each VOC in the total amount produced by herbivore-damaged and undamaged plants can be characterized by the effect values on the y-axis of the PRC plot (Figure 4). Compounds with effect values greater than |0.5| contributed to the overall VOCc blend response, and the greater this value, the more it influences the curve obtained in the plot; i.e., these compounds were deemed responsible for separating treatments (van den Brink & ter Braak, 1999). The volatile profile emitted by herbivore-damaged TB90 plants in the vegetative stage differed from that of undamaged control plants ($F_{1,85} = 6.78$, $P = 0.005$), and 86.5% of the total variance was explained by the treatments and 10.7% by sampling time. The main compounds involved in this differentiation were: β-caryophyllene, (*Z*)-3-hexenyl acetate, myrcene, (*E*,*E*)-α-farnesene, camphene, benzothiazole, DMNT, β-pinene, germacrene D, α-caryophyllene, and *cis*-jasmone (Figure 4). The same trend was observed in the reproductive stage, the chemical profile emitted by herbivory-damaged TB90 plants differed from undamaged plants ($F_{1,79} = 11.18$, $P = 0.005$), and 74.8% of the total variance was explained by the treatments and 16.7% by sampling time. In this case, myrcene, β-caryophyllene, β-pinene, camphene, limonene, α-caryophyllene, (*E*)-β-ocimene, α-copaene, germacrene D, 2-ethylhexan-1-ol, and TMTT were the main compounds that contributed to the divergence (Figure 4).

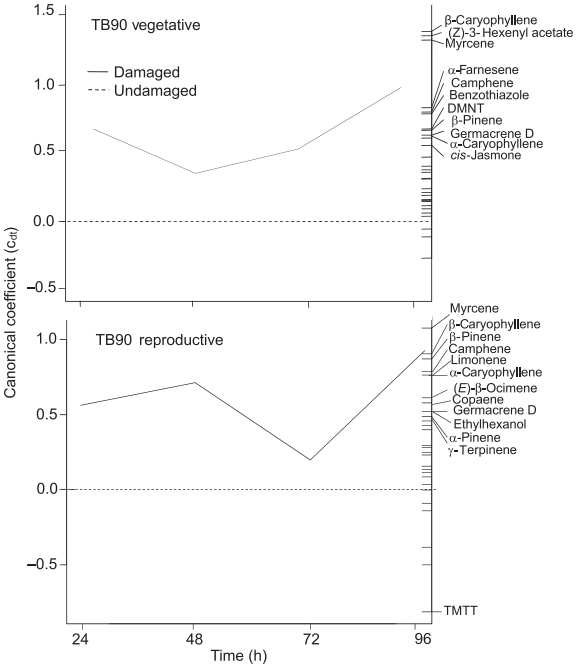

Figure 4 Principal Response Curve (PRC) analyses of volatile blends released by the cotton genotype CNPA TB90 in the vegetative and reproductive stage, as function of sampling time. The lines represent the response patterns of cotton plants to the various treatments.

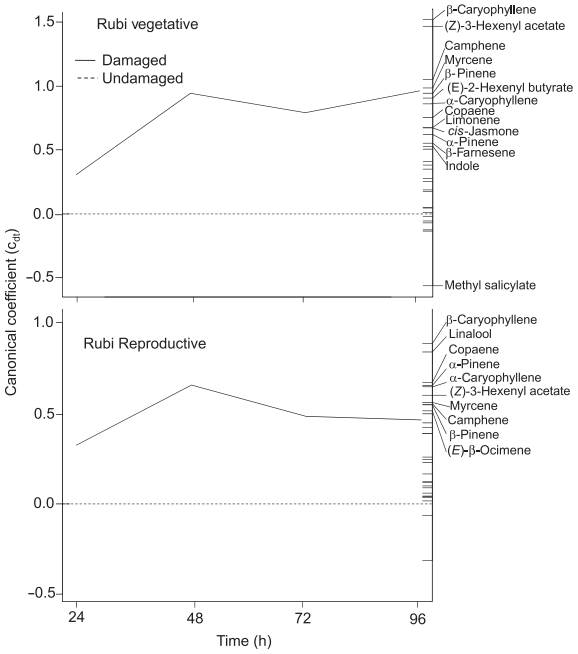

Figure 5 Principal Response Curve (PRC) analyses of volatile blends released by the cotton genotype BRS Rubi in the vegetative stage and reproductive stage, as function of sampling time. The lines represent the response patterns of cotton plants to the various treatments.

For Rubi in the vegetative stage, the chemical profile of volatiles emitted by herbivory-damaged plants differed from that of undamaged plants ($F_{1,72} = 22.17$, $P = 0.005$), with 53.4% of the total variance explained by treatments and 35.5% by sampling time. The main compounds that contributed to the divergence between herbivory-damaged and undamaged Rubi plants in the vegetative stage were: β-caryophyllene, (*Z*)-3-hexenyl acetate, camphene, myrcene, β-pinene, (*E*)-2-hexenyl butyrate, α-caryophyllene, copaene, limonene, *cis*-jasmone, α-pinene, and methyl salicylate (Figure 5). In the reproductive stage the VOC production was higher in herbivore-damaged Rubi plants ($F_{1,88} = 5.98$, $P = 0.01$), and 79.8% of the total variance was explained by treatments and 6.3% by sampling time. In this case, the main VOCs that contributed to the divergence between herbivory-damaged and undamaged plants were: β-caryophyllene, linalool, α-copaene, α-pinene, α-caryophyllene, (*Z*)-3-hexenyl acetate, myrcene, camphene, β-pinene, and (*E*)-β-ocimene (Figure 5).

Boll weevil response to cotton plant volatiles

Adult boll weevil (males and females) were attracted to VOCs released by herbivory-damaged and undamaged cotton plants in the reproductive stage at both 24 and 96 h sampling time of volatile collection. However, there was no significant difference in the weevil response (first choice) between VOCs collected at 24 h for most of the treatments offered, except for the test BRS Rubi herbivore-damaged vs. CNPA TB90 herbivore-damaged ($\chi^2 = 3{,}96$, d.f. = 1, $P = 0.046$). In addition, a significant response (residence time and first choice), which means a preferred choice, only appeared to VOCs collected from plants in the reproductive stage at 96 h. Results for the intermediate time intervals of 48 and 72 h are not shown because most of the time there was no significant difference between treatments offered in the olfactometer.

Adults (males and females) did not show any preference for VOCs collected at 24 h from TB90 and Rubi, herbivore-damaged or undamaged in the reproductive stage, compared between the two nor compared to the control treatment (hexane). This result was confirmed by the weevil's first choice, as well as the residence times in the arms of the olfactometer with the respective treatments. Except for the first choice of males when offered simultaneously VOCs of herbivore-damaged Rubi vs. herbivore-damaged TB90 ($\chi^2 = 3.95$, d.f. = 1, $P = 0.047$), there was an overall preference toward Rubi. On the other hand, in olfactometer bioassays conducted with

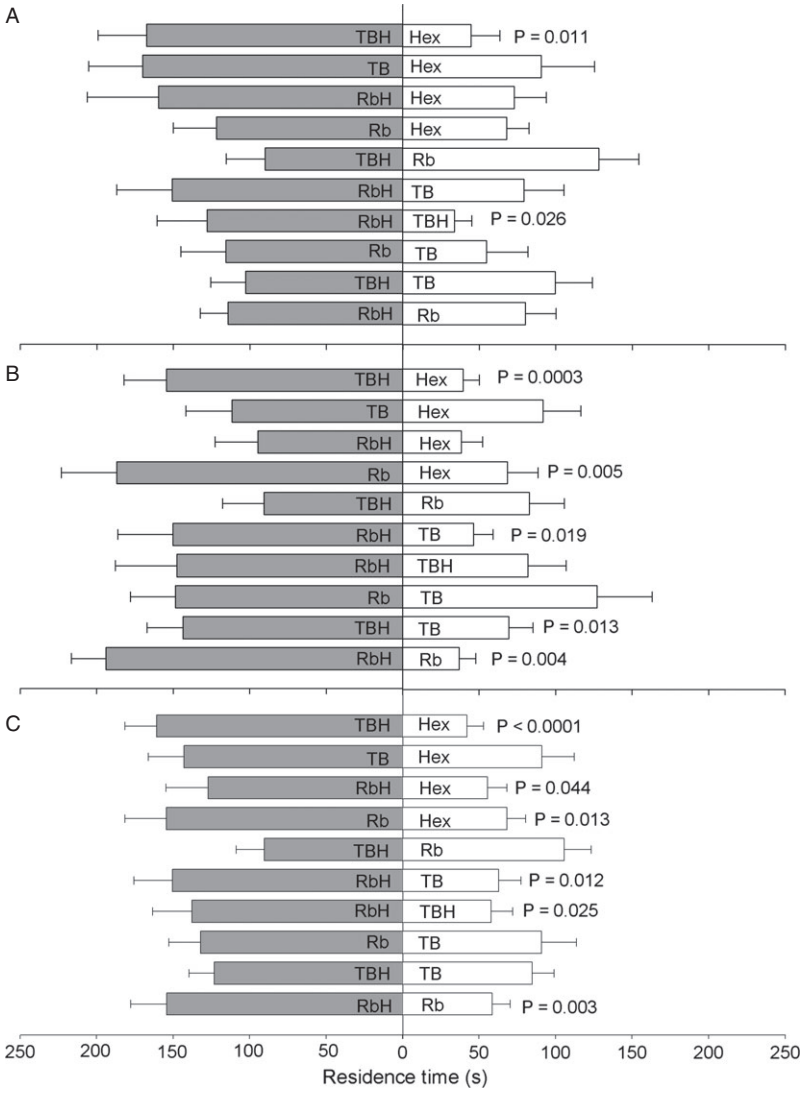

Figure 6 Mean (+ SEM) residence time (s) of (A) *Anthonomus grandis* females (n = 20), (B) males (n = 20), and (C) both (n = 40 insects), to volatile extracts of the cotton genotypes BRS Rubi (Rb) and TB90 (TB), herbivore-damaged (H) or undamaged, and the hexane control (Hex). P-values indicate statistical significance between treatments (paired t-test).

cotton VOCs collected at 96 h, there was a significant difference in response, for both the first choice and total residence time (Figure 6).

Female boll weevils displayed a significant difference in residence time when offered VOCs of herbivore-damaged TB90 vs. hexane control (t = 2.81, d.f. = 1, P = 0.011) and vs. herbivore-damaged Rubi (t = 2.42, d.f. = 1, P = 0.026; Figure 6A). Additionally, the residence time of males was higher in the olfactometer arm with VOCs of herbivore-damaged TB90 compared to undamaged TB90 (t = 2.74, d.f. = 1, P = 0.013) and hexane (t = 3.38, d.f. = 1, P = 0.0032). Similarly, there was also a difference in residence time of males offered VOCs of herbivore-damaged Rubi compared to undamaged Rubi (t = 3.22, d.f. = 1, P = 0.0045) and undamaged TB90 (t = 2.57, d.f. = 1, P = 0.019; Figure 6B).

Analysis of residence times showed that both males and females had a preference for VOCs of herbivore-damaged TB90 (t = 4.34, d.f. = 1, P<0.0001), herbivore-damaged Rubi (t = 2.08, d.f. = 1, P = 0.044), and undamaged Rubi (t = 2.59, d.f. = 1, P = 0.014; Figure 6C) compared to hexane. Overall, adults preferred herbivore-damaged compared to undamaged plants, regardless of the genotype (Rubi: t = 3.13, P = 0.0033; TB90: t = 2.74, P = 0.013, both d.f. = 1).

The data for first choice of boll weevil adults revealed a preference for VOCs of undamaged TB90 (χ^2 = 4.26, d.f. = 1, P = 0.039) and herbivore-damaged TB90 (χ^2 = 6.54, d.f. = 1, P = 0.011) compared to hexane; adults also preferred VOCs of herbivore-damaged Rubi (χ^2 = 5.33, d.f. = 1, P = 0.021) compared to undamaged Rubi or herbivore-damaged TB90. For all other treatment

combinations there was no significant preference (first choice) of boll weevil adults.

Discussion

Previous studies with curculionid beetles have shown that males and females can orient to host plants solely by responding to constitutive plant volatiles in the absence of visual clues (Addesso & McAuslane, 2009). For example, *Anthonomus pomorum* (L.) uses apple plant volatiles to locate the plant (Kalinova et al., 2000). Addesso et al. (2011) showed that males and females of *Anthonomus eugenii* Cano, the pepper weevil, prefer herbivore-damaged *Capsicum annuum* L. plants in the reproductive phenological stage. Olfaction tests conducted in Y-tube olfactometers further showed that adults were more strongly attracted to plants bearing fruits than to plants with flowers, indicating that the pepper weevils are able to discriminate the phenological stage of the host plant. The difference was confirmed by analysis of the bouquet of volatiles released by plants in different developmental stages (Addesso et al., 2011). Magalhães et al. (2012) showed that *A. grandis* also discriminate volatiles emitted by cotton plants in the reproductive vs. the vegetative stage. Together, these studies have shown that curculionid beetles can discriminate the phenological stage of the host plant based solely on the volatiles released, in the absence of any visual or contact chemical clues. Our preliminary tests showed that boll weevil adults did not respond preferentially to volatiles of either cotton genotype (Rubi and TB 90) in the vegetative stage vs. clean air (data not shown). In addition, boll weevil only showed attraction to volatiles of cotton plants in the reproductive stage. This result was expected, as the boll weevil typically colonizes cotton plants only after the appearance of the first cotton squares. Rummel & Curry (1986) suggested that attraction to cotton plants is mediated by volatiles emitted by reproductive structures besides green-leaf volatiles. Therefore, we continued our experiments using only VOCs released by plants in the reproductive stage. However, it should be noted that in the absence of the preferred structures (flowers and squares), the boll weevil can feed on leaves and plant tips (Gilliand & McCoy, 1969), as well as developing bolls (Neves et al., 2013).

Information about insect–plant interactions, especially for key pests such as the boll weevil, has practical implications for research on both cotton resistant genotypes and cotton production. Previous work showed that the cotton genotype TB90 was partially resistant to the boll weevil (Beltrão et al., 2001), but in our laboratory and greenhouse tests we found the contrary. In fact, there was a positive correlation between adult body size and the larger cotton squares produced by TB90 plants, and similar developmental times across all tested cotton genotypes. In addition, oviposition preference trials between TB90 (supposedly resistant) and Rubi (susceptible) cotton genotypes showed no clear preference of the boll weevil for either genotype, suggesting that the boll weevil is able to colonize either one. In the laboratory tests, boll weevil seemed to show some preference for Rubi 1 h after release in the arena, and after some time the preference disappeared. Interestingly, Rubi plants also showed a quantitative (2.4× more) difference in the amount of organic volatiles released in comparison to TB90, although there was no significant difference in number of reproductive structures between the two genotypes. In view of these mixed findings, further conclusions must await more detailed field study and at this time it cannot be definitively stated that the genotype TB90 is less preferred by boll weevil than Rubi.

As expected, the cotton genotype Rubi did not interfere with boll weevil development, nor did TB15, previously shown to be susceptible to *A. grandis* and *Pectinophora gossypiella* (Saunders) (Oliveira et al., 2007). The other cotton genotypes also did not show any significant differences regarding oviposition preference. Therefore, our results suggest that the cotton genotypes studied were all similarly susceptible to the boll weevil as measured by oviposition preferences, developmental time, and weight of emerged adults. Additionally, all genotypes showed a positive correlation between boll weevil fresh body weight and size of cotton squares. This result is consistent with the fact that the boll weevil prefers to lay eggs on squares larger than 6 mm in diameter (Showler, 2005), which contain more nutrients for the developing larvae. Similarly, Michelotto et al. (2007) found that cotton squares with larger diameters produced larger boll weevils. Curiously, weevil development time and weight of cotton squares were not correlated in our study. Because TB85 cotton plants had the smallest effect on the relation of square size to boll weevil size produced, as well as causing prolonged developmental time relative to the other genotypes, further study is warranted and results suggest that there may be some kind of negative effect of this genotype on the boll weevil.

Our findings showed that boll weevils responded positively to VOCs released by cotton plants of the genotypes TB90 and Rubi, and that they preferred the volatiles emitted by herbivory-damaged plants compared to volatiles emitted by undamaged plants. In addition, boll weevil feeding on plants caused an increase in the total amount of VOCs produced, consistent with previous studies on other plants showing that elevated VOCs were released in response to herbivore damage from both specialists and

generalists (Rodriguez-Saona et al., 2003; Moraes et al., 2011; Magalhães et al., 2012).

Among the several compounds that influence the curves on PRC analysis of both genotypes evaluated, β-caryophyllene appeared consistently as an important compound with higher effect in both genotypes and both physiological stages evaluated. In addition, the compounds myrcene and (*Z*)-3-hexenyl acetate appeared to strongly influence the outcome of PRC analysis. These compounds might be important in the boll weevil's response to airborne extracts from herbivory-damaged cotton plants. β-Caryophyllene was produced in higher amounts in the vegetative stage of herbivory-damaged compared to undamaged plants. Likewise, the compound myrcene was produced in higher amounts by herbivory-damaged plants of both genotypes and in both physiological stages evaluated compared to undamaged plants. Similar results were seen for (*Z*)-3-hexenyl acetate from Rubi in both physiological stages and TB90 in the vegetative stage. Electrophysiological investigation of the olfactory responses of boll weevil adults to these compounds is potentially rewarding.

Overall, both sexes showed similar responses to VOCs released by cotton plants. Even though herbivory-damaged plants potentially provoke increased intraspecific competition, weevils were more attracted to volatiles released by damaged plants. These results corroborate those from Magalhães et al. (2012), who also found that boll weevil adults did not respond to VOCs released by plants fed upon by non-host-specific insects, such as *Euschistus heros* (Fabricius) and *Spodoptera frugiperda* (JE Smith). Their findings suggest that potential synergistic effects due to induced VOCs resulting from other herbivorous insects feeding on cotton will probably not affect boll weevil interactions with cotton.

One boll weevil female is able to oviposit 7–11× a day (Lloyd, 1986), and more than one oviposition can be done in the same cotton square. However, many times the boll weevil female only feeds on the square, especially if it is not large enough to support larval development. This fact was verified in our results in the laboratory and greenhouse. One herbivore-damaged plant releasing volatiles indicates its presence and phenological stage, but not necessarily the presence of conspecifics developing in the same plant. Thus, from the perspective of a gravid boll weevil female, it is better to respond to those volatiles than not, as they indicate a potential host plant nearby, even though it may be already occupied by conspecifics.

Studies investigating the attraction of the boll weevil to VOCs released by cotton plants are of major importance for understanding the interaction between pest and host plant, clarifying dispersal behavior, or for improving methods to monitor and control this pest. Thus, plant VOCs are promising tools for managing insect pests (Collatz & Dorn, 2013). For example, Nehme et al. (2010) found an 85% increase in insect attraction when testing plant volatiles combined with the aggregation pheromone of the Asian beetle *Anoplophora glabripennis* (Motschulsky). As suggested by Dickens (1990), the volatiles β-caryophyllene, (*E*)-2-hexen-1-ol, (*E*)-β-ocimene, and β-bisabolol can be used as synergists with the aggregation pheromone of the boll weevil to increase the number of insects captured in a pheromone trap. Even though some of these compounds were not found in the genotypes tested in our study, some of the volatiles released by the genotypes we tested are likely involved in the attraction of boll weevils to cotton plants. Magalhães et al. (2012) found an increase in boll weevil attraction to its aggregation pheromone when enriched with volatiles of the cotton cultivar Delta Opal after herbivory.

A review of studies conducted with different cotton genotypes reveals considerable variability in the chemical profile of volatiles released, mainly in the quantities of the compounds (Röse et al., 1998; Magalhães et al., 2012). For example, the Delta-Opal cultivar evaluated by Magalhães et al. (2012) released a higher amount of volatiles compared to the amounts released by the cultivars in our study. Based on our results, which largely concur with those of previous studies, we can conclude that cotton VOCs can modulate the behavior of boll weevils, including functioning as attractants to this pest. Additionally, the genotypes TB90 and TB85, presumably resistant, tested in this study against the susceptible Rubi and TB15, did not show a negative impact in the development of the boll weevil to characterize host plant resistance. Therefore, this similar susceptibility among tested genotypes could not lead to a reduction in cotton field colonization by the boll weevil. This is the first report of biological and behavioral boll weevil data for the cotton genotypes studied, which can serve as support information for further research and cotton management practices in the field.

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