Influence of Two Acyclic Homoterpenes (Tetranorterpenes) on the Foraging Behavior of Anthonomus grandis Boh

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Abstract Previous studies have shown that the boll weevil, Anthonomus grandis, is attracted to constitutive and conspecific herbivore-induced cotton volatiles, preferring the blend emitted by cotton at the reproductive over the vegetative stage. Moreover, this preference was paralleled by the release of the acyclic homoterpenes (tetranorterpenes) (E)-4,8-dimethyl-1,3, 7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3, 7,11-tetraene (TMTT) in Delta Opal cotton being higher at the vegetative than at the reproductive stage. Here, we evaluated whether this difference in release of acyclic homoterpenes also occurred in other cotton varieties, and if boll weevils could recognize these compounds as indicators of a specific cotton phenological stage. Results showed that cotton genotypes CNPA TB-90, BRS-293 and Delta Opal all produced higher levels of DMNT and TMTT at the vegetative stage than at the reproductive stage and that these homoterpenes allowed for principal component analysis separation of volatiles produced by the two phenological stages. Electroantennograms con-firmed boll weevil antennal responses to DMNT and TMTT. Behavioral assays, using Y-tube olfactometers, showed that adding synthetic homoterpenes to reproductive cotton vola-tiles (mimicking cotton at the vegetative stage in terms of homoterpene levels) resulted in reduced attraction to boll wee-vils compared to that to unmodified reproductive cotton. Weevils showed no preference when given a choice between plants at the vegetative stage and the vegetative stage-mimicked plant. Altogether, the results show that DMNT and TMTT are used by boll weevils to distinguish between cotton phenological stages.

Keywords Cotton · Homoterpenes · Host plant · Phenological stages · Plant volatiles · Ontogenetic · Coleoptera · Curculionidae

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Introduction

Plant volatiles mediate important trophic interactions, particularly between plants and their herbivores. These volatiles provide crucial cues for phytophagous insects to locate suitable host plants upon which to feed or oviposit (Bruce and Pickett 2011). In addition, herbivores often can exploit these compounds to obtain information, for example, concerning the presence of competitors and potential natural enemies, plant quality, and phenology (Addesso et al. 2011; Magalhães et al. 2012; Tasin et al. 2011).

Changes in volatile production can occur as plants develop through their life cycle (Hare 2010). For herbivores, the use of associated volatile composition of a specific ontogenetic stage allows them to locate the most suitable host plant phenology. Rapid host location is important for exploiting ephemeral food resources (Schwarz et al. 2009). Within the subfamily Anthonominae (Curculionidae), some species can distinguish host plant phenology based solely on emitted volatiles: Anthonomus eugenii Cano prefers fruiting over flowering pepper volatiles (Addesso et al. 2011), Anthonomus pomorum (L.) distinguishes apple tree flower buds at different pheno-logical stages (Kalinová et al. 2000), and Anthonomus grandis

Boheman prefers reproductive over vegetative cotton volatiles (Magalhães et al. 2012).

Anthonomus grandis, the boll weevil, is the main pest on cotton crops in the Neotropical region. The boll weevil eats and oviposits on cotton squares and bolls. In Brazil, farmers have adopted heavy spraying with insecticides to control this pest. The aggregation pheromone of this insect is used to monitor populations on the crop. However, when cotton reaches the reproductive stage, the number of insects captured in traps baited with pheromone is drastically reduced and, as a result, weevils go straight to plants (Rummel and Curry 1986). Cotton phenology, therefore, has an important role in A. grandis population dynamics, and chemical cues have an ac-tive role in this process. By responding to specific volatile blends from the preferred ontogenetic host plant stage, boll weevils can migrate from refuge areas to suitable structures for feeding and ovipositing.

Previously, we showed that adult boll weevils were attracted to undamaged and conspecific herbivore-induced volatiles, preferring the blend emitted by cotton at the reproductive stage over that emitted by the vegetative stage (Magalhães et al. 2012). Nevertheless, the compounds used by boll weevils for differentiating specific cotton stages have not yet been elucidated. Magalhães et al. (2012) reported that a major difference in the chemical profile of volatiles emitted from vegetative and reproductive undamaged cotton (cv. Delta Opal) was the amount of the acyclic homoterpenes or, more correctly tetranorterpenes, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), with greater amounts being released at the vegetative stage. To test the hypothesis that DMNT and TMTT are directly related to differentiation of host plant phenological stage by the boll weevil, we examined the electrophysiological and behavioral responses of adult A. grandis to these compounds. We also investigated whether different cotton genotypes release different DMNT and TMTT amounts at distinct phenological stages.

Methods and Materials

Insect Rearing Boll weevils were reared in plastic containers on an artificial diet [a mixture of agar, beer yeast, wheat germ, soy protein, glucose, ascorbic and sorbic acid, Nipagin, flour from embryo cottonseed (Pharmamedia®, Traders Protein, USA), Wesson salt mixture, Vanderzant's vitamin and water; Schmidt et al. 2001] under controlled conditions (25 ± 1 °C, 60 ± 10 % RH, and 14:10 L:D). Newly emerged adults were sexed by the tergal-notch method (Sappington and Spurgeon 2000), transferred to 250 ml plastic cages (25 insects/cage), and allowed to feed on artificial diet. Food and water were changed three times per week. To prevent interactions between sexes, males were kept in separated cages from females after the imaginal moult. Virgin 10-day-old male and female weevils were used in all experiments.

Plants Gossypium hirsutum L. (genotypes CNPA TB-90, BRS-293 and Delta Opal) were grown individually in 1.5 L pots filled with soil and an organic substrate (in a proportion of 1:1). Plants were grown in a greenhouse under controlled conditions (27 ± 1 °C and 14:10 L:D). Cotton plants used in experiments were 6 weeks old at the vegetative stage (up to 6 expanded true leaves and about 30 cm high) and 12 weeks old at the reproductive stage (presence of squares).

Air Entrainment of Plants Cotton plants, at vegetative and reproductive stages, were placed individually in cylindrical glass chambers (internal volume 10 L). Plastic pots and soil were covered by aluminum foil to reduce the collection of volatiles from these sources. Twelve independent chambers were run simultaneously. Charcoal-filtered air was pumped in at 1.0 $l.min^{-1}$ and drawn out at 0.6 $l.min^{-1}$ through an adsorbent Super Q tube (60 mg, 80–100 mesh, Alltech, PA, USA), connected to the system via PTFE tubing. The difference in flow created a slight positive pressure to ensure that unfiltered air did not enter the system. Plant volatiles were collected for 24 hr before the adsorbent tubes were eluted with 0.5 ml of redistilled hexane. As an internal standard, 1 µl of 16-hexadecanolide (in distilled hexane) was added to the samples, at a final concentration of 0.01 $mg.ml^{-1}$. Six plants of each variety were entrained at the vegetative and reproductive stages. Samples were stored in vials at −20 °C until used for experiments.

Gas Chromatography (GC) Volatiles were analyzed on an Agilent 7890A equipped with a flame ionization detector (FID) and a non-polar DB-5MS column (60 m × 0.32 mm i.d., 0.25 µm film thickness, Supelco, PA, USA). Oven temperature was maintained at 50 °C for 2 min, then increased at 5 $°C.min^{-1}$ to 180 °C, held for 0.1 min., then increased at 10 $°C.min^{-1}$ to 250 °C, and held for 20 min. The FID was set at 270 °C and the injector at 250 °C. One microliter of each sample was injected into a splitless injector, with helium as carrier gas. Data were collected with EZChrom Elite software. Generalized Linear Model (GLM) and Deviance analysis with gamma distribution and inverse as link functions were used to compare the total amount of released homoterpenes from vegetative and reproductive stages. The statistical analyses were carried out using R Statistical Software (Foundation for Statistical Computing). To evaluate the influence of all compounds in separating cotton phenological stages, principal component analysis (PCA) was applied to the multivariate data. The PCA was performed using a correlation matrix and comparison between two groups (vegetative and reproductive). The PCA was carried out using Paleontological Statistics Software (PAST version 3.10).

Coupled Gas Chromatography/Mass Spectrometry (GC/MS) Identifications were performed on an Agilent 5975MSD quadrupole mass spectrometer, equipped with a DB-5MD column (30 m × 0.32 mm i.d., 0.25 μm film, Supelco, PA, USA), a splitless injector, and helium as carrier gas. Ionization was by electron impact (70 eV, source temper-ature at 200 °C). The injector was at 250 °C, and the column oven was programmed using the same temperature program as in the GC-FID analyses. Data were collected using Agilent ChemStation software. Tentative identifications were made by comparison of spectra with mass spectral library databases (NIST, 2008) and through use of retention indices (RIs), and identities confirmed by co-injection of air entrainment samples with authentic standards. Retention indices were calculated using the retention times of a series of linear hydrocarbon al-kanes (C_8–C_{24}) (Lucero et al. 2009).

Chemicals Hexane for HPLC (≥97 % redistilled) was purchased from Sigma-Aldrich (Steinheim, Germany). (E)-4,8-Dimethyl-1,3,7-nonatriene and trimethyl-1,3,7,11-tridecatetraene were synthesized from geraniol and (E,E)-farnesol, respectively (Leopold 1990). α-Pinene (98 %), camphene 90 %, β-pinene (99 %), myrcene (90 %), (Z)-3-hexenyl acetate (98 %), (E)-3-hexenyl butyrate (98 %), ocimene (90 %), benzaldehyde (99 %), indole (98.5 %), methyl salic-ylate (99 %), α-copaene (90 %) and alloaromadendrene (90 %) were purchased from Sigma Aldrich (Steinheim, Germany). Linalool, α-humulene (96 %), β-caryophyllene (80 %) and limonene (97 %) were purchased from TCI-America (Portland, OR, USA). Geranylacetone (96 %) was purchased from TCI (Tokyo, Japan).

Olfactometer Bioassays Behavioral assays of 10-day-old adult male and female boll weevils to vegetative and repro-ductive cotton (cv. Delta Opal) and synthetic volatile blends of DMNT and TMTT were carried out using a Y-tube olfactometer. The following experiments were run (the quantities used are described in Table 1): A) synthetic blend of homoterpenes + plant background; (1) cotton at the reproductive stage ($Plant^{Rep}$) vs. cotton at the vegetative stage ($Plant^{Veg}$), (2) $Plant^{Rep}$ + synthetic blend of DMNT and TMTT at the same concentration and proportion as in the air entrainment of vegetative cotton (Mix^{Veg}) vs. $Plant^{Veg}$, (3) $Plant^{Veg}$ + Mix^{Veg} vs. $Plant^{Veg}$, (4) $Plant^{Rep}$ + Mix^{Veg} vs. hexane. B) Synthetic blends of homoterpenes; (5) Mix^{Veg} vs. hexane, (6) synthetic blend of DMNT and TMTT at the same concentration and proportion as in the air entrainment of reproductive cotton (Mix^{Rep}) vs. hexane, (7) Mix^{Veg} vs. Mix^{Rep}. C) single homoterpenes; (8) DMNT at the same concentration as in the air entrainment of vegetative cotton ($DMNT^{Veg}$) vs. hexane, (9) DMNT at the same concentration as in the air entrainment of reproductive cotton ($DMNT^{Rep}$) vs. hexane, (10) $TMTT^{Veg}$ vs. hexane and (11) $TMTT^{Rep}$ vs. hexane.

Table 1 Amounts (ng/hr) of the acyclic homoterpenes, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), released by cotton (cv. Delta Opal) in vegetative and reproductive stage air entrainment samples

Compound name	Vegetative	Reproductive
DMNT	136.0	29.4
TMTT	246.4	66.5

Details of the olfactometer and bioassay procedures are described by Magalhães et al. (2012). A polyethylene tere-phthalate (PET) oven bag (250 × 380 mm, Sainsbury's, UK) was placed carefully over a plant and sealed around the stem using wire. In one of the top corners, a hole was made to accommodate silicon tubing connecting the plant to a glass syringe and to the olfactometer. Prior to use, the oven bags were baked at 180 °C for 2 hr. Filter papers containing 10 μl of the synthetic blends of DMNT and TMTT were placed inside the glass syringes. Charcoal filtered and humidified air was pumped in at 0.6 $L.min^{-1}$ and drawn out at 0.2 $l.min^{-1}$. Weevils were starved for 24 hr prior to bioassays, and a single boll weevil was introduced at the base of the Y-tube olfactometer. The weevil was observed for 10 min., and the first choice and residence time (the time spent in an arm) noted. Each weevil was used only once, and the filter paper and cotton plants replaced after five replicates. Both sexes were bioassayed until a total of 50 males and 50 females had responded. After five repetitions, the Y-tube olfactometer and the side on which the treatment was presented was swapped to avoid any positional bias. Data analysis of the first choice of the boll weevil was carried out by logistic regression and Wald's Chi-square test to assess significance (R Statistical Software). Residence times in treatment and control arms were analyzed by Paired-t tests (R Statistical Software).

Electrophysiological Responses of the Boll Weevil to Homoterpenes Electroantennogram (EAG) recordings were made using Ag-AgCl glass electrodes filled with Ringer solution. A boll weevil antenna was excised and mounted between the electrodes. The extreme tips of the scape and flagellum were cut off with a microscalpel to ensure good contact. The stimulus delivery system employed a piece of filter paper in a disposable Pasteur pipette. The stimuli were delivered over the preparation in a constant 1 $L.min^{-1}$ airstream and applied (2 sec duration) at 30 sec intervals. Ten microliter aliquots of standard solutions of DMNT and TMTT (1 $mg.ml^{-1}$ in distilled hexane) were applied to strips of filter paper, with the solvent being allowed to evaporate for 60 sec before the strip was placed into the pipette. The hexane control was tested before and after each test compound, and an average was taken. The homoterpenes were presented in random order. Responses to test compounds were compared to the average

of responses to hexane for each replicate. Antennae from 10 female and 10 male boll weevils were tested. EAG responses were normalized to an artificial 0.1 mV signal and were recorded using specialized software (EAG for Windows, 1999, Syntech, The Netherlands). The responses of weevils to control and test compounds were analyzed using analysis of variance (ANOVA) and the means were compared using Tukey's 95 % confidence test (GenStat 17th edition).

Results

Air Entrainment Analysis Chemical analyses of the air entrainment samples showed that the total amount of homoterpenes (DMNT and TMTT) differed between vegeta-tive and reproductive cotton (Fig. 1). The PCA analysis showed that these were the main compounds responsible for separating volatiles of cotton phenological stages in the three genotypes evaluated (Fig. 2). Homoterpene production was higher at the vegetative stage than at the reproductive stage for all three genotypes (CNPA TB-90: ANODEV$\chi 2$ = 36.136, df = 1, P < 0.001; BRS-293: ANODEV$\chi 2$ = 5.565, df = 1, P = 0.01; Delta Opal: ANODEV$\chi 2$ = 14.442, df = 1, P < 0.001) (Fig. 1). The amounts of DMNT and TMTT were not different among genotypes at the reproductive stage (ANODEV$\chi 2$ = 1.948, df = 2, P = 0.377), but at the vegetative stage they were different among genotypes (ANODEV$\chi 2$ = 11.729, df =2, P = 0.003).

Behavioral Responses in the Y-Tube Olfactometer The quantities of homoterpenes present in cotton plant headspace samples on which the synthetic blends for bioassays were

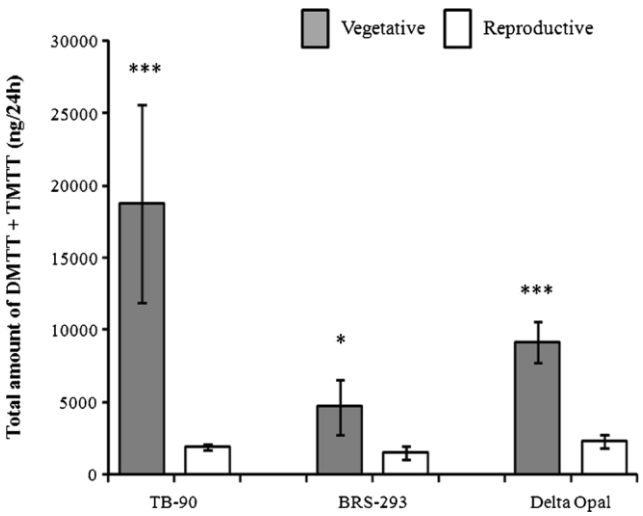

Fig. 1 Total amount (ng/hr) of the acyclic homoterpenes, (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), emitted by CNPA TB-90, BRS-293 and Delta Opal cotton at vegetative and reproductive stages. In each genotype, asterisks represent differences between phenological stages (* 0.05 > P > 0.01, ** 0.01 > P > 0.001, *** P < 0.001)

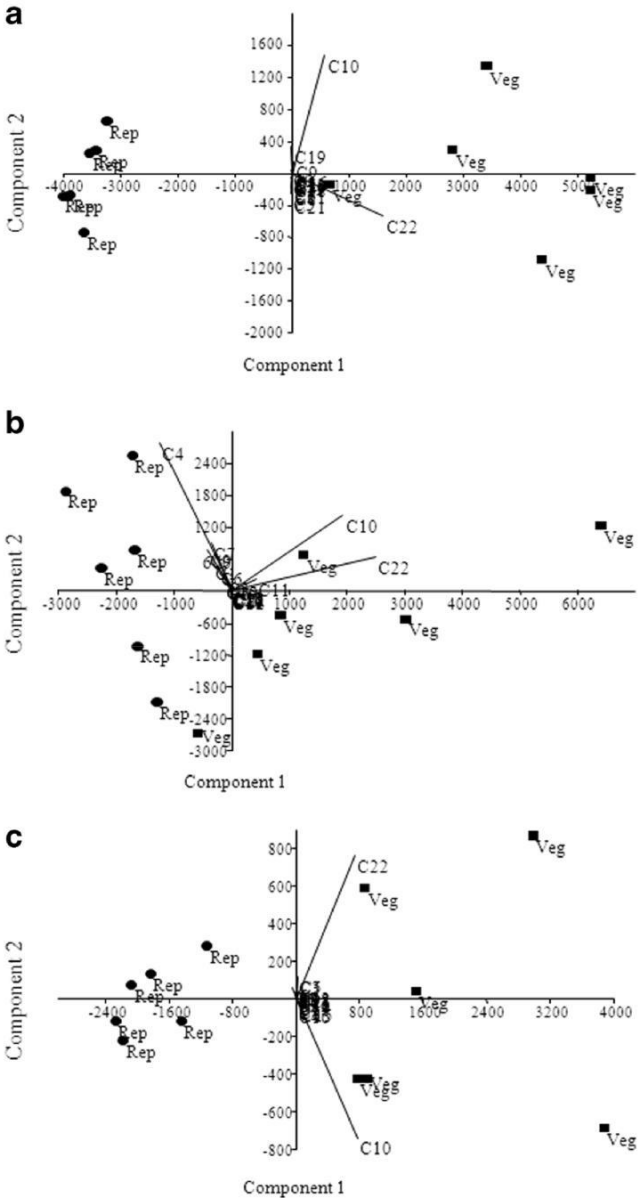

Fig. 2 Principal component analysis (PCA) ordination for components 1 and 2 of volatile compounds emitted by undamaged cotton plants at the vegetative and reproductive stages from three different genotypes: a CNPA TB-90, b BRS-293 and c Delta Opal. The compared groups were plants at the vegetative (Veg) and reproductive (Rep) stages. C corresponds to volatile compounds: C4 = β-myrcene, C10 = (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and C22 = (E,E)-4,8, 12-trimethyltrideca-1,3,7,11-tetraene (TMTT)

based are listed in Table 1. When the volatiles from cotton at the vegetative stage ($Plant^{Veg}$) were compared against volatiles from cotton at the reproductive stage ($Plant^{Rep}$), weevils preferred the volatiles from $Plant^{Rep}$ in first choice (males $\chi 2$ = 7.54, df = 1, P = 0.006 and females $\chi 2$ = 4.28, df = 1, P = 0.038) and residence time (males t = 2.068, df = 49, P = 0.043 and females t = 2.457, df = 49, P = 0.017) (Figs. 3 and 4). When the volatiles from $Plant^{Veg}$ were compared against volatiles from $Plant^{Rep}$ + the synthetic blend of

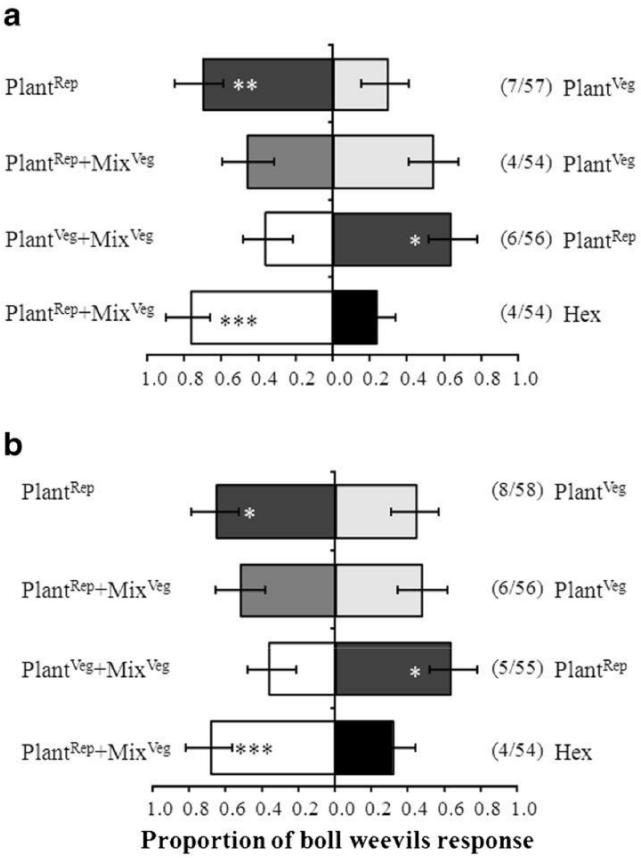

Fig. 3 First choice of male (a) and female (b) boll weevils in a Y-tube olfactometer. $Plant^{Rep}$ = reproductive cotton; $Plant^{Veg}$ = vegetative cotton; Mix^{Veg} = synthetic blend of (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) at the same concentration and proportion as in the air entrainment of vegetative cotton; $Plant^{Rep}$ + Mix^{Veg} = cotton at the reproductive stage + Mix^{Veg}; $Plant^{Veg}$ + Mix^{Veg} = cotton at the vegetative stage + Mix^{Veg}; and Hex = hexane. Asterisks indicate differences (* 0.05 > P > 0.01, ** 0.01 > P > 0.001, *** P < 0.001) between pairs of treatments. Bars indicate 95 % confidence intervals. Numbers in parentheses indicate insects that did not respond to either treatment, and the number of bioassays done

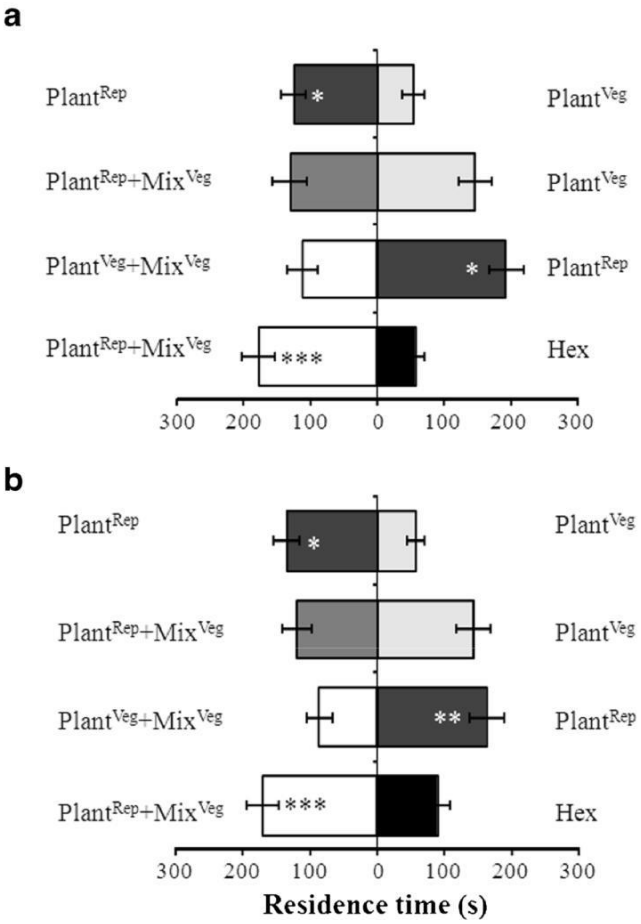

Fig. 4 Mean residence time (seconds) of male (a) and female (b) boll weevils in a Y-tube olfactometer. $Plant^{Rep}$ = reproductive cotton; $Plant^{Veg}$ = vegetative cotton; Mix^{Veg} = synthetic blend of (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) at the same concentration and proportion as in the air entrainment of vegetative cotton; $Plant^{Rep}$ + Mix^{Veg} = cotton at the reproductive stage + Mix^{Veg}; $Plant^{Veg}$ + Mix^{Veg} = cotton at the vegetative stage + Mix^{Veg}; and Hex = hexane. Asterisks indicate differences (* 0.05 > P > 0.01, ** 0.01 > P > 0.001, *** P < 0.001) between pairs of treatments. Bars indicate standard errors

DMNT and TMTT at the same concentration and proportion as in the air entrainment sample from vegetative cotton (Mix^{Veg}), i.e., a reproductive plant mimicking a vegetative plant in terms of homoterpene levels, weevils did not show any preference in either first choice (males $\chi 2 = 0.32$, df = 1, P = 0.572 and females $\chi 2 = 0.08$, df = 1, P = 0.777) or resi-dence time (males $t = -0.342$, df = 49, P = 0.733 and females $t = -0.659$, df = 49, P = 0.512) (Figs. 3 and 4). The weevils showed a preference for cotton at the reproductive stage ($Plant^{Rep}$) over the $Plant^{Rep}$ + Mix^{Veg} (mimic of a vegetative plant) in both first choice (males $\chi 2 = 3.81$, df = 1, P = 0.05 and females $\chi 2 = 3.81$, df = 1, P = 0.05) (Fig. 3) and residence time (males $t = -2.057$, df = 49, P = 0.045 and females $t = -2.296$, df = 49, P = 0.025) (Fig. 4). Both male and female weevils showed preference for the mimicked vegetative plant over the hexane control in both first choice (males $\chi 2 = 12.12$, df = 1, P < 0.001 and females $\chi 2 = 6.18$, df = 1, P = 0.012) and residence time (males $t = 3.956$, df = 49, P < 0.001 and females $t = -2.629$, df = 49, P = 0.011) (Figs. 3 and 4, respectively).

Only female boll weevils showed a preference for Mix^{Veg} over hexane in first choice ($\chi 2 = 6.18$, df = 1, P = 0.012) and residence time ($t = 2.62$, df = 49, P = 0.001) (Figs. 5 and 6, respectively). In contrast, when the synthetic blend of DMNT and TMTT at the same concentration and proportion as found in the air entrainment of reproductive cotton (Mix^{Rep}) was used, female boll weevils did not show a preference in either first choice ($\chi 2 = 0.72$, df = 1, P = 0.397) or residence time ($t = 1.54$, df = 49, P = 0.12) (Figs. 5b and 6b, respectively), but males spent more time in the treated area compared to the control ($t = 2.136$, df = 49, P = 0.037) (Fig. 6a). However, when Mix^{Veg} and Mix^{Rep} were compared, both male and female weevils preferred the Mix^{Rep} treated area to the Mix^{Veg} area in first choice (males $\chi 2 = 6.18$, df = 1, P = 0.012 and females

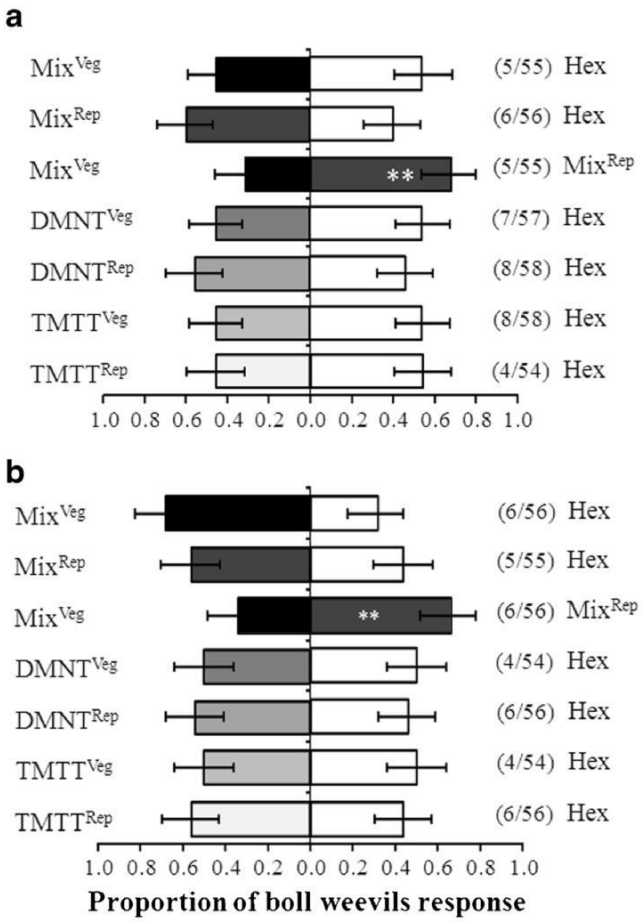

Fig. 5 First choice of male (a) and female (b) boll weevils in a Y-tube olfactometer. Mix^{Veg} = synthetic blend of (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) at the same concentration and proportion as in the air entrainment of vegetative cotton; Mix^{Rep} = synthetic blend of DMNT and TMTT at the same concentration and proportion as in the air entrainment of reproductive cotton; $DMNT^{Veg}$ = DMNT at the same concentration as in the air entrainment of vegetative cotton; $DMNT^{Rep}$ = DMNT at the same concentration as in the air entrainment of reproductive cotton; $TMTT^{Veg}$ = TMTT at the same concentration as in the air entrainment of vegetative cotton; $TMTT^{Rep}$ = TMTT at the same concentration as in the air entrainment of reproductive cotton; and Hex = hexane. Asterisks indicate differences (* $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $P < 0.001$) between pairs of treatments. Bars indicate 95 % confidence intervals. Numbers in parentheses indicate insects that did not respond to either treatment, and the number of bioassays done

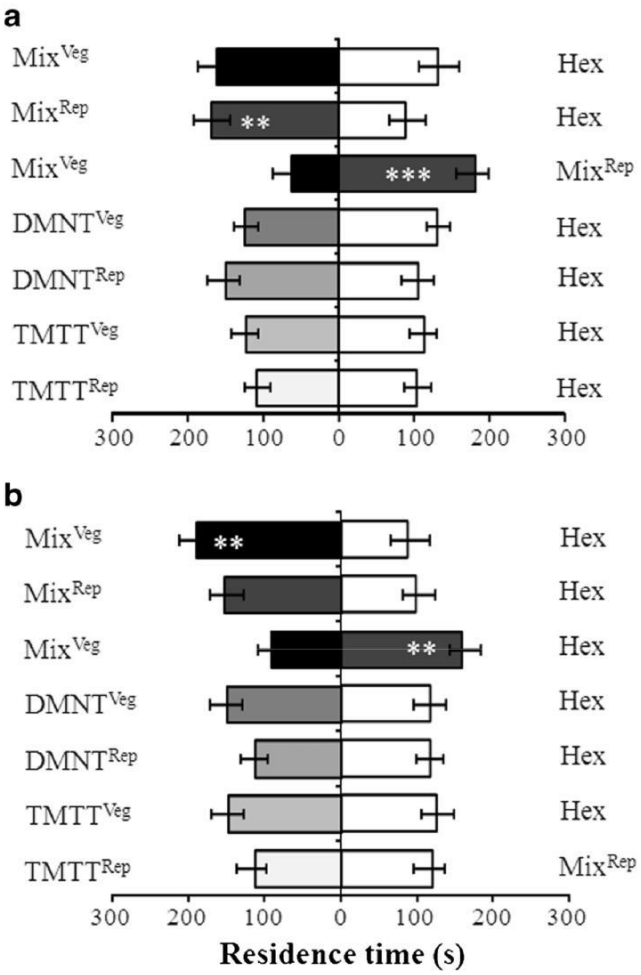

Fig. 6 Mean residence time (seconds) of male (a) and female (b) boll weevils in a Y-tube olfactometer. Mix^{Veg} = synthetic blend of (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) at the same concentration and proportion as in the air entrainment of vegetative cotton; Mix^{Rep} = synthetic blend of DMNT and TMTT at the same concentration and proportion as in the air entrainment of reproductive cotton; $DMNT^{Veg}$ = DMNT at the same concentration as in the air entrainment of vegetative cotton; $DMNT^{Rep}$ = DMNT at the same concentration as in the air entrainment of reproductive cotton; $TMTT^{Veg}$ = TMTT at the same concentration as in the air entrainment of vegetative cotton; $TMTT^{Rep}$ = TMTT at the same concentration as in the air entrainment of reproductive cotton; and Hex = hexane. Asterisks indicate differences (* $0.05 > P > 0.01$, ** $0.01 > P > 0.001$, *** $P < 0.001$) between pairs of treatments. Bars indicate standard errors

$\chi 2 = 4.94$, df = 1, $P = 0.026$) (Fig. 5). The same pattern was observed for residence time, with weevils spending more time in the Mix^{Rep} area (males $t = 3.71$, df = 49, $P < 0.001$ and females $t = 2.10$, df = 49, $P = 0.04$) over that in Mix^{Veg} area (Fig. 6). When the individual compounds DMNT and TMTT, at concentrations representing both vegetative and reproductive stages, were evaluated, boll weevils did not differentiate them from the hexane ($P > 0.05$) (Figs. 5 and 6).

Electrophysiological Responses of Boll Weevils to the Homoterpenes Responses of male and female A. grandis to DMNT and TMTT elicited different electrophysiological re-sponses at 1 $mg.ml^{-1}$ (Fig. 7). EAG responses did not differ between male and female weevils.

Discussion

In this study, cotton genotypes CNPA TB-90, BRS-293 and Delta Opal released 9-, 3- and 6-fold more, respectively, of the acyclic homoterpenes DMNT and TMTT at the vegetative

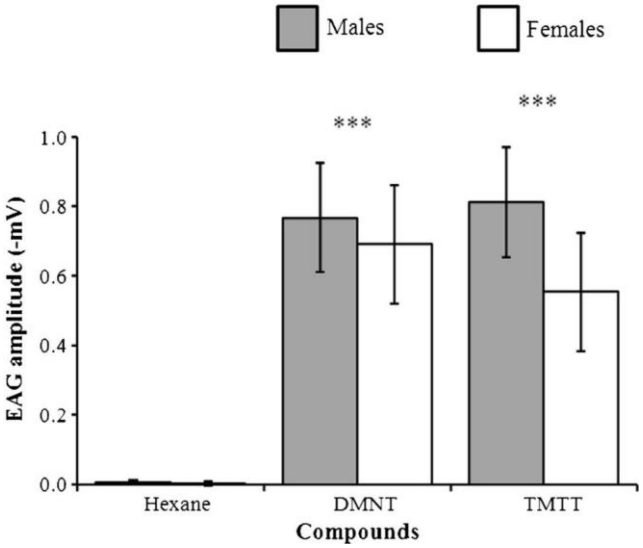

Fig. 7 Electroantennogram (EAG) (−mV ± SE) responses of male and female Anthonomus grandis antennae to the acyclic homoterpenes (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (E , E)-4,8,1 2-trimethyltrideca-1,3,7,11-tetraene (TMTT) (N = 10). Responses were normalized to a 0.1 mV signal. Asterisks indicate difference between the test compounds and control ($P < 0.001$)

stage than at the reproductive stage. The PCA analysis showed that these two homoterpenes explained the differences in vol-atile profiles between the two phenological stages. The other compounds released by cotton included mainly sesquiterpenes and monoterpenes, but did not change quantities as the plants developed as drastically as did the homoterpenes. In BRS-293, the amount of the monoterpene β-myrcene changed in accord with the change from vegetative to reproductive stage; however, changes in this compound were not observed in the other genotypes.

We used manipulative experiments to test the role of DMNT and TMTT plus plant volatiles in the differentiation of vegetative and reproductive cotton. Adding synthetic homoterpenes to reproductive cotton volatiles made the plants considerably less attractive to the boll weevil compared to the unmodified reproductive cotton volatiles. By manipulating the amounts of homoterpenes, we showed that male and female boll weevils preferred cotton (i.e., in the reproductive stage) that released lower amounts of acyclic homoterpenes. Thus, the quantity of these homoterpenes appears important for dif-ferentiation of cotton phenological stage by boll weevils.

DMNT and TMTT are biosynthesized as a consequence of oxidative stress resulting in loss of four carbons associated with the tertiary alcohol group of higher terpene alcohols, and are common volatiles emitted by a myriad of plants (Tholl et al. 2011). These compounds have been reported as components of floral odors of night-scented plant species and plant foliage, serving different ecological functions in plant/ arthropod and plant/plant interactions. Several studies have indicated a role of these homoterpenes in indirect plant defense, attracting natural enemies of herbivores, such as the predatory mite Phytoseiulus persimillis Athias-Henriot to lima bean (de Boer et al. 2004), the parasitic wasp Cotesia sesamiae (Cameron) to maize (Tamiru et al. 2011), and the egg parasitoid Trichogramma bournieri Pintureau & Babault to maize (Tamiru et al. 2011). Moreover, TMTT may play a role in plant/plant activation of defense genes in lima bean, making the plant more attractive to predatory mites (Arimura et al. 2000). As well as an indirect defense role, DMNT and TMTT also have been reported as a playing a role in direct plant defense, repelling aphids from cotton (Hegde et al. 2011) and the leafhopper Cicadulina storey from maize (Oluwafemi et al. 2011). The function of DMNT and TMTT in plants goes beyond defense: they can be exploited by herbivores as attrac-tive signals, as demonstrated for the black vine weevil, Otiorhynchus sulcatu Fabricius (van Tol et al. 2012), the tea weevil, Myllocerinus aurolineatus (Voss) (Sun et al. 2012), and the strawberry blossom weevil, A. rubi (Bichão et al. 2005). Despite being frequently emitted by plants upon feed-ing damage (Tholl et al. 2011), DMNT and TMTT are also emitted constitutively by undamaged plants, such as the mo-lasses grass, Melinis minutiflora P. Beauv. (Poalaes: Poaceae) (Khan et al. 1997), maize, Zea mays L. (Poales: Poaceae) (Hoballah et al. 2004), and cotton (Magalhães et al. 2012; Rose et al. 1996).

In cotton, the production of these compounds is increased by herbivore damage by different insect species, such as A. grandis, Euschistus heros (Fabricius) and Spodoptera frugiperda (J.E. Smith) (Magalhães et al. 2012), Aphis gossypii Glover (Hegde et al. 2011), Spodoptera exigua (Hübner) and Bemisia tabaci (Gennadius) (Rodriguez-Saona et al. 2003), Heliothis virescens (Fabricius) and Helicoverpa zea (Boddie) (Rose and Tumlinson 2004). Interestingly, even in herbivore-damaged cotton at the reproductive stage, the production of acyclic homoterpenes is lower, or at an amount similar to that of undamaged cotton at the vegetative stage (Magalhães et al. 2012), supporting our hypothesis that the boll weevil uses these homoterpenes to differentiate host phenological stage.

Magalhães et al. (2012) had previously shown that vegetative cotton releases higher quantities of total volatiles, but not a qual-itatively different blend, to that of reproductive cotton. Ontogenetic-driven changes in plants may affect secondary me-tabolite dynamics and the interaction of herbivores with their hosts (Barton and Hanley 2013). Depending on the physiologi-cal properties at each ontogenetic stage, the levels of secondary metabolites may be limited by growth or by reproduction, de-creasing or being reallocated to important reproductive tissues. The decreased release of acyclic homoterpenes in cotton at the reproductive stage may be a direct result of reallocation of re-sources to squares and bolls (cotton reproductive structures). As different classes of terpenoids in cotton are biosynthetically re-lated (Optiz et al. 2008), it is possible that chemical protection of these reproductive tissues becomes more important than

protection of vegetative tissue. Therefore, the reduction in the emission of DMNT and TMTT at the reproductive stage could result from the synthesis of involatile terpenoids, such as terpenoid aldehydes, used to protect the newly formed squares and bolls for overall fitness. In flowering Nicotiana attenuata Torr., there is a reallocation of phenylpropanoid-polyamine conjugates (compounds related to the increase of plant resistance) from vegetative to reproductive tissues (Kaur et al. 2010). In addition, the production of the phytohormones ethylene and jasmonate, associated with direct defense, decreases as plants age and reach the reproductive stage (Diezel et al. 2011).

EAG recordings showed that the antennae of A. grandis responded to both DMNT and TMTT, with the responses of male and female boll weevil to the synthetic compounds being similar. An absence of sexual dimorphism in the response to plant-derived volatiles also has been found for the strawberry blossom weevil, A. rubi (Bichão et al. 2005), and the apple blossom weevil, A. pomorum (Kalinová et al. 2000).

Overwintering boll weevils stay in sheltered areas surrounding cotton fields and feed on pollen, mainly from plants in the Smilacaceae (Ribeiro et al. 2010). Cotton plants, especially at the reproductive stage, are highly attractive to boll weevils, and the migration of weevils from natural refuges to cotton fields starts with squaring cotton (Rummel and Curry 1986). During this stage, pheromone-baited traps are less efficient, and the number of weevils captured is drastically reduced (Rummel and Curry 1986). Presumably, the decrease in homoterpene amounts at the reproductive stage of cotton indicates the availability of a food source and oviposition site to the boll weevil.

Although this study demonstrated the influence of the acy-clic homoterpenes DMNT and TMTT on the differentiation of cotton ontogeny by the boll weevil, it is unclear whether these compounds play a role in actual host location by boll weevils. In the Y-tube olfactometer, DMNT or TMTT alone, or in mix-ture, at both vegetative or reproductive plant concentrations, did not consistently attract adult A. grandis when tested against hexane. As has been shown in other systems (e.g., Bruce and Pickett 2011), host attraction can involve a complex mixture of volatiles. Identification of the blend of compounds responsible for attraction of boll weevils to reproductive cot-ton currently is underway in our laboratory. The ultimate pur-pose is to formulate a host plant-derived volatile blend that could be used to increase the effectiveness of the current boll weevil monitoring system, greatly reducing the amount of insecticides used in cotton crops (Oliveira et al. 2014).

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