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Ineffective weapon – the role of plant secondary metabolites in cotton defence against the boll weevil

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Abstract:	Plant cultivar selection for resistance to herbivore pests is an effective, environmentally safe and inexpensive method to implement in integrated pest management programs. In this study, we evaluated seven cotton genotypes with respect to the production of volatile organic compounds (VOCs) and non-volatile compounds (terpenoid aldehydes (TAs)), and the attraction and feeding preference of adult boll weevils. Chemical analyses of VOCs from BRS-293, BRS-Rubi, CNPA TB-15, CNPA TB-85, CNPA TB-90, Delta Opal, and Empire Glandless showed that there were few qualitative and quantitative differences across the range of genotypes. In contrast, major differences in TA content were observed, with CNPA TB-15 and CNPA TB-85 producing higher levels of TAs compared to the other genotypes. Our results showed that boll weevil attraction and feeding behaviour was not positively or negatively influenced by the terpenoid content (volatile and non-volatile compounds) of cotton genotypes. The results in this study suggest that boll weevils have adapted physiologically to cope with cotton chemical defence mechanisms.
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Ineffective weapon – the role of plant secondary metabolites in cotton defence against the boll weevil

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Main conclusion

Cotton genotypes emit similar VOC profiles but contain different levels of TAs. Boll weevils do not display any preference in attraction and feeding behaviour towards particular cotton genotypes, suggesting that they are physiologically adapted to cope with cotton chemical defence mechanisms.

Abstract

Plant cultivar selection for resistance to herbivore pests is an effective, environmentally safe and inexpensive method to implement in integrated pest management programs. In this study, we evaluated seven cotton genotypes with respect to the production of volatile organic compounds (VOCs) and non-volatile compounds (terpenoid aldehydes (TAs)), and the attraction and feeding preference of adult boll weevils. Chemical analyses of VOCs from BRS-293, BRS-Rubi, CNPA TB-15, CNPA TB-85, CNPA TB-90, Delta Opal, and Empire Glandless showed that there were few qualitative and quantitative differences across the range of genotypes. In contrast, major differences in TA content were observed, with CNPA TB-15 and CNPA TB-85 producing higher levels of TAs compared to the other genotypes. Our results showed that boll weevil attraction and feeding behaviour was not positively or negatively influenced by the terpenoid content (volatile and non-volatile compounds) of cotton genotypes. The results in this study suggest that boll weevils have adapted physiologically to cope with cotton chemical defence mechanisms.

Keywords: VOCs, terpenoid aldehydes, plant defence, *Gossypium hirsutum, Anthonomus grandis*.

Introduction

Many plant secondary metabolites have a role in defence against herbivores and pathogens, however, their production is costly to plants (Dudareva et al. 2013). Plant survival against herbivore damage is achieved through the development of resistance mechanisms. Thus, resistance is related to characteristics that make plants less susceptible to herbivores and can be broadly classified as (i) antixenosis, often referred to as non-preference, a plant-expressed trait that has adverse effects on insect behaviour; (ii) antibiosis, traits that negatively impact herbivore biology; and (iii) tolerance, the ability of a plant to withstand herbivory without any decline in yield (Painter 1951). Inducible defences are advantageous to plants and have evolved to reduce production costs because they are generated only upon herbivore damage (feeding and oviposition) (Aljbory and Chen 2018). The cost-saving benefit of inducible defence becomes risky depending on the frequency and intensity of attacks that a plant undergoes. Therefore, plants that are likely to be attacked by herbivores should have low levels of inducible defences and high levels of constitutive defences (Zangerl and Rutledge 1996; Wittstock and Gershenzon 2002).

Cotton, *Gossypium hirsutum* L., attracts a diverse complex of arthropod species for shelter and food (Hagenbucher et al. 2013), but at the same time possesses a large array of defence traits against herbivores (Sadras and Felton 2010; Stipanovic et al. 2010). A great variety of terpenes are produced and stored in subepidermal pigment glands present in different cotton organs (Optiz et al. 2008). Higher levels of terpenoids are found in young leaves, squares, and bolls (Bell et al. 1978). These organs accumulate several terpenoid products, including mono-, homo- and sesquiterpenes, and terpenoid aldehydes (TAs) (Stipanovic et al. 2010). Modern varieties of cotton usually produce the monoterpenes α-pinene, β-pinene, myrcene, limonene, and (*E*)-ocimene, whereas γ-terpinene is found in primitive cotton races (Bell et al., 1995). Most varieties produce the sesquiterpenes α-copaene, α-humulene, (*E*)-caryophyllene, δ-cadinene, γ-bisabolene and aromadendrene (Stipanovic et al. 2010).

The irregular acyclic homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) are also found in modern cotton genotypes (Röse et al. 1996; Magalhães et al. 2012, 2016). The compounds gossypol, hemigossypolene, and the heliocides (H1, H2, H3, and H4) are among the most widespread TAs in glanded varieties of cotton (Bell 1986; Stipanovic et al. 2010).

The effects of cotton terpenoids on insect herbivores are highly variable and depend on the degree of herbivore specialization. Several terpenoids are known to adversely affect the development and survival of generalist herbivores, such as the caterpillars *Helicoverpa armigera* (Hübner) and *H. zea* (Boddie) (Lepidoptera: Noctuidae) (Stipanovic et al. 2006; Kong et al. 2010). On the other hand, specialist herbivores, such as the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), have adapted physiologically to cope with cotton defence mechanisms (Stipanovic et al. 2010). Adult boll weevils use cotton volatile organic compounds (VOCs), including terpenoid compounds, to locate host plants (Magalhães et al. 2012, 2016, 2018). Terpenoid aldehydes, such as gossypol, have slight oviposition stimulating effects (Hedin and McCarty 1990). Gossypol-rich diets tend to produce larger weevils and a high percent of egg hatch compared to low gossypol diets (Linding et al. 1979). The evolution of such offensive traits has relied upon a long evolutionary history of the boll weevil with cotton plants, dating the domestication of cotton in Central America, as a result of an initial host exchange, migrating from wild to cultivated cotton species (Jones 2001).

Cultivar selection for resistance to herbivore pests is an effective, environmentally safe and inexpensive method to implement in integrated pest management programs. Previous experiments have shown that cotton genotypes CNPA TB-85 and CNPA TB-90 suffer less attack by the boll weevil, exhibiting a certain degree of resistance (Beltrão et al. 2001); and the genotypes BRS-293, BRS-Rubi, CNPA TB-15, Delta Opal, and Empire Glandless are attacked more heavily, in the particular context of each experimental setup (Jenkins et al. 1967; Busoli et al. 2007; Oliveira et al. 2007; Silva et al. 2015). The genotypes CNPA TB-15, CNPA TB-85, and CNPA TB-90 resulted from the crossing of PNH_3 (early maturing) with three other lines (T-227-2-6, T-326-1, and T-1180W) that were generated from the crossing of Stoneville 213 with Mexican landraces (T-277, T-326, and T-1180) (Carvalho et al. 1996). In this study, we investigated the production of VOCs and TAs in different cotton genotypes and evaluate resistance traits of the genotypes towards the boll weevil. Specifically, we asked the following questions: (i) are there differences in VOCs/TAs content between cotton genotypes; (ii) do boll weevils show preference to certain cotton genotypes based on odour perception; (iii) how do differences in TAs content affect boll weevil feeding preference for certain genotypes?

Material and methods

Insect rearing

Boll weevils, *A. grandis*, 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 moulted adults were sexed using the tergal-notch method (Sappington and Spurgeon 2000), transferred to 250 mL plastic container (15 insects/container), and fed with an artificial diet. Food and water were changed three times per week. To prevent interactions between sexes, males were kept in containers separated from females after the imaginal moult. In all experiments, 10-d-old virgin weevils were used.

Plants

Cotton seeds from the genotypes BRS-293, BRS-Rubi, CNPA TB-15, CNPA TB-85, CNPA TB-90, and Empire Glandless were obtained from the Active Germplasm Bank of Embrapa Algodão, while the seeds from Delta Opal were donated by MDM-Maeda Delta Pine Monsanto. Plants were grown individually in 1.5 L pots filled with soil (3:1:1:0.03:0.03 red-yellow Latosol, sand, organic manure, fertilizer, and limestone). Plants were grown in a greenhouse under controlled conditions (27 ± 1 °C and 14:10 L: D, and the light intensity of 95.600 lx) and watered every second day. Plants used in the experiments were 12-wk-old and at the reproductive stage (presence of squares).

Dynamic headspace collection

Plants were placed individually in a cylindrical glass chamber (internal volume 10 L). Cotton VOCs were collected from six plants of each genotype. The plastic pots and soil were covered with aluminium 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 $L \cdot min^{-1}$ and drawn out at 0.6 $L \cdot min^{-1}$ through a Porapak Q (60 mg, 80-100 mesh, Supelco, PA, USA) trap, 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. Cotton VOCs were collected for 24 h and the adsorbent traps were eluted with 0.5 mL of redistilled hexane. Samples were stored in vials at -20 °C until used in chemical analyses and behavioural bioassays.

Gas Chromatography (GC-FID) analysis

Cotton VOCs were analysed on an Agilent 7890-A equipped with a flame ionization detector (FID) and a non-polar DB-5MS column (60 m x 0.32 mm i.d., 0.25 μm film thickness, Supelco, PA, USA). The oven temperature was maintained at 50 °C for 2 min, programmed at 5° $C.min^{-1}$ to 180 °C, held for 0.1 min, then 10 $°C.min^{-1}$ to 250 °C, and held for 20 min. The FID was at 270 °C and the injector at 250 °C. As an internal standard, 1 μL of 16-hexadecanolide was added to the samples with a final concentration of 9.8 $\mu g \cdot mL^{-1}$. One microliter of each sample was injected on a splitless injector, with helium as the carrier gas. Data were collected with GC Open Lab.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Tentative identifications of collected cotton VOCs were performed on an Agilent 5975-MSD quadrupole mass spectrometer coupled to a gas chromatograph (Agilent 7890A) equipped with a DB-5MS column (30 m x 0.25 mm i.d., 0.25 μm film, Supelco, PA, USA), a splitless injector and helium as the carrier gas. Ionization was by electron impact (70 eV, source temperature at 200 °C). The injector was at 250 °C using the same temperature programme as in GC-FID analysis. Data were collected with ChemStation software. Identifications were made by comparison of spectra with mass spectral library databases (NIST 2008) and use of retention indices (RI), and were confirmed by co-injection of the air entrainment sample with authentic standards. The RI were calculated by comparison to the retention times of a series of linear hydrocarbon alkanes (C_8 –C_{24}) analysed with the same separation method. Cotton VOCs were classified according to chemical classes as: monoterpenes [α-pinene, camphene, β-pinene, myrcene, limonene, (*E*)-ocimene, γ-terpinene, and linalool], homoterpenes [DMNT and

TMTT], sesquiterpenes [(*E*)-caryophyllene, α-humulene, δ-guaiene, δ-cadinene, and nerolidol], esters [(*Z*)-3-hexenyl acetate and methyl salicylate] and ketones [geranylacetone].

Extraction of TAs

Terpenoid aldehydes were extracted from young leaves fully opened, large squares (5-8 mm), and young bolls (2-3 cm diameter) for all tested genotypes (*n*=6 for each structure). As a control, Empire Glandless cotton was used as it does not produce any TA. Young leaves, squares, and bolls were excised from the stem with a razor blade and immediately placed on liquid nitrogen, freeze-dried for 48 h, and then ground. Cotton TAs were extracted according to the protocol described by Benson et al. (2001). The ground fine powder was passed through a 90 µm sieve, and 100 mg of the material was extracted with 10 mL of acetonitrile/water/phosphoric acid (80:20:0.1) by ultrasonic extraction during 3 min. The samples were centrifuged for 3 min at 2800*g* and filtered through a 0.45 µm pore size membrane (Minisart, Sartorius Stedim Biotech). The supernatant was transferred to glass vials and stored at 4 ºC until use.

High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analyses

Reversed-phase HPLC analysis was performed on a Shimadzu LC-20AD (Shimadzu Corporation, Kyoto, Japan) coupled to a Photodiode Array Detector (Shimadzu SPD-M20A PDA) set at 272 nm. A 20 µL injection was made on a HiChrom ACE-5 C18 column (250 x 4.6 mm; HiChrom Ltd., Reading, UK). The column was eluted with acetonitrile (solvent A) and 0.5% formic acid in water (solvent B), with the following gradient: 75% A (0.01 min) – 75% A (35 min) – 95% A (40 min) – 95% A (60 min) – 75% A (65 min) – 75% A (85 min). The solvent flow rate was 1.0 $L \cdot min^{-1}$ held constant. All samples were checked by triplicate injections. Cotton TAs concentrations were quantified in terms of gossypol equivalents (McAuslane et al., 1997), because hemigossypolene (HGQ) and heliocides (H1-4) standards were not commercially available. The LC-MS/MS analysis was performed using a Micromass Quattro Ultima quadrupole mass spectrometer hyphenated to a Waters Acquity UPLC system. The MS was operated in negative ion mode, with a capillary voltage of 2.7 kV, cone voltage 50 eV, and mass range 50-1000 m/z. The source temperature was 130 ºC, desolvation temperature 350 ºC, desolvation gas flow 1000 $L \cdot h^{-1}$ (Nitrogen), and cone gas flow 60 $L \cdot h^{-1}$ (Nitrogen). Samples were injected via the Acquity sample manager, injecting 2 µL onto two Acquity UPLC BEC C18 columns (1.7 µm, 2.1 x 50 mm and 1.7 µm, 2.1 x 150 mm). The solvents used were the same as in the HPLC analysis, but here we used three gradient modes: (i) 70% A over 10.8 min, 95% A over 15.1 min, and 75% A over 2.1 min; (ii) 70% A over 30 min, 95% A over 42 min, and 70% A over 6 min; and (iii) 70% A over 49.9 min, 95% A over 70 min, and 75% A over 10,1 min. The run time and flow rate were 28 min at 0.58 $ml \cdot min^{-1}$, 78 min at 0.21 $ml \cdot min^{-1}$, and 130 min at 0.21 $ml \cdot min^{-1}$, respectively. Gossypol was identified by comparing the retention time to that of commercially available standard. The compounds HGQ and H1-4 were identified comparing the retention time and mass spectra obtained in our study (Figure 1, supplementary material) with previously published data (Stipanovic et al. 1988; Altman et al. 1990; Benson et al. 2001).

Chemicals

Authentic chemical standards of α-pinene (98%), camphene (90%), β-pinene (99%), myrcene (90%), (*E*)-ocimene (90%), γ-terpinene (97%), and methyl salicylate (99%) were purchased from Sigma-Aldrich Inc. (Steinheim, Germany). Gossypol from cotton seeds (95%) was purchased from Sigma-Aldrich Inc. (St. Louis, USA). Linalool (96%), α-humulene (96%), (*E*)-caryophyllene (90%), and limonene (97%) were purchased from TCI-America (Portland, USA), and geranylacetone (96%) from TCI-Japan (Tokyo, Japan). The solvent hexane (>97% redistilled) was purchased from Sigma-Aldrich Inc. (Steinheim, Germany), whilst acetonitrile (99%) and phosphoric acid (85%) were purchased from Fisher Scientific (Leic., UK). (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) (95%) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) (97%) were synthesized from geraniol and (*E,E*)-farnesol, respectively (Leopold 1990).

Olfactometer bioassays

A four-choice olfactometer was used to test olfactory responses of 10-d-old virgin adult male and female boll weevils. A square acrylic block, with an X-shaped cavity sandwiched between two glass plates, was used as the bioassay arena. The trunk of the apparatus was 37 cm long and each arm, 25 cm long. Filter papers containing 5 µL of the air entrainment samples (equivalent to the volatiles released by one plant in ~1 h) were placed inside glass syringes connected to the arms of an olfactometer via silicone tubing. Charcoal-filtered humidified air was pumped in at 1.2 $L \cdot min^{-1}$ and drawn out at 0.4 $L \cdot min^{-1}$. Weevils were starved for 24 h before bioassays. A single weevil was introduced at the centre of the four-choice olfactometer and was observed for 10 min, and the first choice (when it moved >2 cm into the arm) and residence time (the time spent in an arm) were noted. Each weevil was used only once, and the filter paper was replaced after three replicates. Assays were continued until a total of 40 individuals had responded (positive chemotaxis). After six repetitions, the four-choice olfactometer and the side on which the odours were presented were rotated to avoid any positional bias. The bioassays were divided into two groups: the first group compared cotton genotypes with similar terpenoid relative proportion (≥95%) (CNPA TB-15, CNPA TB-85, CNPA TB-90, and Delta Opal), and the second group, genotypes with more divergent terpenoid relative proportion (≤95%) (BRS-293, BRS-Rubi, Delta Opal, and Empire Glandless). As we had an odd number of genotypes, Delta Opal (commercial genotype) was present in both groups.

Closed arena bioassays

A closed arena was used to determine the feeding preference of 10-d-old virgin adult male and female boll weevils. The assays were carried out under laboratory conditions (25 ± 1°C and 14:10 L:D) using 15 and 10 cm diameter Petri dishes (first and second sets of bioassays, respectively). The weevils were starved for 24 h before the experiment to encourage immediate feeding. In all experiments, 5-9 mm square buds with removed bracteoles were used. In the first set of bioassays, one square bud of each cotton genotype was placed equidistantly in the Petri dish. A total of 30 weevils (males and females) was assayed. The second set of bioassays was carried out to evaluate the feeding preference regarding the presence and absence of TAs. Thus, cotton square buds of the glandless genotype (Empire Glandless, no TA) were compared with each of the remaining glanded genotypes. A total of 11 males and 11 females was assayed. A single boll weevil was placed into the centre of the arena and after 48 h and the number of feeding punctures in each square bud was registered.

Statistical analyses

The estimation of the percentage of each class of compound was obtained by calculating the ratio between their concentration and the total concentration of detected VOCs of each genotype. A Generalized Linear Model (GLM) and Deviance analysis with gamma distribution and inverse as link function were used to compare the total amount of released VOCs and TAs from the different genotypes. When the analysis showed significant effects among the genotypes, means were compared using contrast analyses. Data analysis of the first choice of weevils was performed using the Kruskal-Wallis test to assess significance to random choices (25% of choices for each arm of the olfactometer). Residence time in treatment and control arms was analysed by Friedman's test. The number of feeding punctures was analysed using ANOVA and treatment means were separated using Tukey's test. The statistical analyses were performed using RStudio, Inc. (version 0.99.903).

Results

GC-FID and GC-MS analyses

Chemical analyses of VOCs collected from seven cotton genotypes revealed few qualitative differences, apart from Empire Glandless which emitted fewer compounds (Table 1). The following major compounds were identified by GC-MS and RI comparison with authentic standards as: α-pinene (RI=938), camphene (RI=954), β-pinene (RI=981), myrcene (RI=990), (*Z*)-3-hexenyl acetate (RI=1005), 1-decyne (RI=1024), limonene (RI=1033), (*E*)-ocimene (RI=1050), γ-terpinene (RI=1063), linalool (RI=1104), DMNT (RI=1114), methyl salicylate (RI=1193), (*E*)-caryophyllene (RI=1424), geranylacetone (RI=1449), α-humulene (RI=1461), δ-guaiene (RI=1505), δ-cadinene (RI=1520), nerolidol (RI=1556), and TMTT (RI=1575). There was no genotype-specific VOCs and most compounds were detected in all genotypes (Table 1). The VOC profiles were also quantitatively similar across the genotypes (Figure 1), apart from Empire Glandless which produced the lowest levels of total VOCs (ANODEV χ^2=18.36, df=6, P=0.005). Despite the low variability in total VOCs, there were significant differences for compound classes across the genotypes (Figure 2). CNPA TB-90 and Empire Glandless produced the lowest level of monoterpenes compared to the other genotypes (ANODEV χ^2=50.43, df=6, P<0.001) (Figure 2-A). There was no significant difference in the amounts of sesquiterpenes (ANODEV χ^2=8.38, df=6, P=0.21) (Figure 2-B), but there were significant differences in the amounts of homoterpenes (ANODEV χ^2=19.63, df=6, P=0.003) (Figure 2-C). For the esters, BRS-293 produced the highest level across all the genotypes (Figure 2-D), especially methyl salicylate (Table 1). BRS-293 emitted approximately 300 ng.day^{-1} of methyl salicylate, whereas the other genotypes emitted no more than 90 ng.day^{-1} (Table 1). Comparing the other genotypes, there was no difference in the total amount of esters emitted (P>0.05) (Figure 2-D). The total amount of ketones was also different across genotypes (ANODEV χ^2=19.63, df=6, P=0.003) (Figure 2-E). Taken together, the results showed that the terpenoids were the major components in the VOCs of cotton genotypes, and the homo- and monoterpenes had the highest relative proportion in BRS-Rubi, CNPA TB-15, CNPA TB-85, CNPA TB-90, Delta Opal, and Empire Glandless. For BRS-293, methyl salicylate (ester) was also a major compound (Figure 3).

HPLC analysis

Cotton leaves, squares, and bolls contained different amounts of the TAs hemigossypolene, gossypol and heliocides (H1, H2, H3, and H4) (Table 2). As expected, Empire Glandless did not produce any TA (Table 2). The highest levels of TAs in leaves (ANODEV χ^2=320.33, df=5, P<0.001) and squares (ANODEV χ^2=295.52, df=5, P<0.001) were produced by CNPA TB-15 and CNPA TB-85 (Figure 4-A, B). In the bolls, BRS-293 and

279 CNPA TB-15 (ANODEV χ^2=370.99, df=5, P<0.001) produced the highest levels of TAs (Figure 4-C). The
280 genotypes CNPA TB-90 and Delta Opal had similar amounts of TAs in all three cotton structures, whereas BRS-
281 Rubi had different amounts of TAs in leaves, squares, and bolls (Figure 4).
282
283 **Bioassays**
284 In four-choice bioassays with adult male and female boll weevils, there was no preference in the first choice of
285 weevils for the first group (CNPA TB-15, CNPA TB-85, CNPA TB-90, and Delta Opal) (males: Kruskal-Wallis
286 ANOVA χ^2=0.637, P=0.769; females: χ^2=1.403, P=0.769) and the second group of genotypes (BRS-291, BRS-
287 Rubi, Delta Opal, and Empire Glandless) (males: Kruskal-Wallis ANOVA χ^2=0.149, P=0.966; females: χ^2=0.603,
288 P=0.966). Similar results were obtained for the residence time, i.e., no difference in the time spent in each arm of
289 the olfactometer for the first group (males: Friedman ANOVA χ^2=0.411, df=3, P=0.833; females: χ^2=0.676, df=3,
290 P=0.766) and the second group of genotypes (males: Friedman ANOVA χ^2=0.060, df=3, P=0.994; females:
291 χ^2=16.275, df=3, P=0.480). There was no difference in the mean number of feeding punctures of males
292 (1.36±0.32; ANOVA F=0.65, P=0.68) and females (2.54±0.56; ANOVA F=0.78, P=0.59) in cotton squares when
293 all genotypes were tested together. When each glanded genotype was tested individually against the glandless
294 genotype in feeding experiments, there was no difference in the mean number of feeding punctures (P>0.05).
295
296 **Discussion**
297 Chemical analyses of VOCs from cotton genotypes BRS-293, BRS-Rubi, CNPA TB-15, CNPA TB-85, CNPA
298 TB-90, Delta Opal, and Empire Glandless showed that there were few qualitative and quantitative differences. On
299 the other hand, major differences in TAs were observed across the genotypes, in which CNPA TB-15 and CNPA
300 TB-85 produced higher levels of these compounds than the other evaluated genotypes. Empire Glandless produced
301 as expected lower levels of volatile and non-volatile terpenoids. Boll weevils did not display any preference in
302 attraction and feeding behaviour regarding the terpenoid content of the cotton genotypes.
303 Terpenoids in cotton are formed from biosynthetically-related intermediate skeletons via the
304 condensation of the five-carbon monomer isopentenyl diphosphate (IPP) and its isomer, dimethylallyl
305 diphosphate (DMAPP) (Davis and Essenberg 1995; Optiz et al. 2008; Chen et al. 2011). Two distinct biosynthetic
306 pathways produce these five-carbon monomers: the mevalonate (MVA) pathway which provides the precursors
307 for the biosynthesis of sesquiterpenes, homoterpenes, and TAs, and the methylerythritol 4-phosphate (MEP)
308 pathway, the predominant source of monoterpenes (Cheng et al. 2007; Chen et al. 2011). Although there is
309 subcellular compartmentalization in MVA and MEP pathways, metabolic cross-talk between these two routes can
310 occur, allowing them both to produce all classes of terpenoids (Rodriguez-Concepción 2006; Optiz et al. 2014).
311 For example, addition of an IPP unit to the precursor of monoterpenes (geranyl diphosphate, GPP) forms the
312 precursor of the sesquiterpenes (farnesyl diphosphate, FPP) (Gershenzon 2010). FPP is also the precursor of the
313 TAs, and by Diels-Alder-type reactions between hemigossypolene and myrcene or (*E*)-ocimene (monoterpenes),
314 for example, form the heliocides H2-H3 and H1-H4, respectively (Stipanovic 1992). Here, the genotype Empire
315 Glandless did not produce detectable levels of myrcene and low levels of (*E*)-ocimene, which could be associated
316 with the absence of heliocides, providing evidence for the interaction of biosynthetic pathways.
317 The VOCs identified in the seven cotton genotypes are largely in accordance with those obtained from
318 previous studies (Röse et al. 1996; Paré and Tumlinson 1998; Rodriguez-Saona et al. 2003; Moraes et al. 2011;

Magalhães et al. 2012; Silva et al. 2015). The overall similarities in the relative composition of VOC classes are reflected in the non-preference of boll weevils to any of the tested genotypes. Non-preference for BRS-Rubi and CNPA TB-90 in response to cotton VOCs, possibly due to the similarity in the composition of the VOC blend responsible for the boll weevil's attraction, has already been reported (Silva et al. 2015). Thus, cotton VOCs from the seven genotypes do not seem to be involved in the resistance to the boll weevil or, as the weevil is well adapted to the plant, VOCs no longer interfere in this process. Similar to other studies, the modern genotypes studied here also did not produce, or produced in small amounts, the monoterpene γ-terpinene (Bell et al. 1995; Loughrin et al. 1995). Non-volatiles, on the other hand, had a great deal of variation in the proportion of TAs. Hemigossypolone and heliocides were the major TAs in leaves, squares, and bolls. Previous studies using different cotton genotypes also showed that in leaves gossypol is found in low amounts, and that hemigossypolone and heliocides comprise 90% of total TAs (Stipanovic et al. 1978, 1988; Altman et al. 1989; Optiz et al. 2008). In squares, this percentage changes and gossypol corresponds to 45% of total TA content (Stipanovic et al. 1988; Altman et al. 1989). This pattern was observed for Delta Opal only, as the other genotypes contained hemigossypolone and heliocide as the major TA. In the bolls, heliciodes are the major TA (Stipanovic et al. 1988) and our results corroborate this finding. Usually, gossypol is the only TA found in seeds (Stipanovic et al. 1988), however, we did not evaluate seed content. Empire Glandless was the only genotype that did not produce any TA.

Boll weevils did not show a feeding preference for squares from the different tested genotypes. Despite the differences in the composition of TAs, the variability found seems not to affect boll weevil feeding behaviour even when comparing genotypes with different levels of TAs with a glandless genotype that does not produce any TA. These data agree with previous studies showing that the boll weevil has no preference for varieties of glandless and normal-glanded cotton but prefers high-gossypol varieties (Jenkins et al. 1967; Singh and Weaver 1972). The literature is inconclusive on the importance of TAs for boll weevil nutrition. Some studies show that gossypol does not act as a phagostimulant (Hedin et al. 1966; Maxwell et al. 1966) and that monosaccharides, on the other hand, stimulate adult feeding (Hardee and Mitchel 1997). Previous studies using the same genotypes showed that the boll weevils had no preference for BRS-Rubi and CNPA TB-90 (Silva et al. 2015), but preferred FMT-701, FMT-910 and FMX-993 over Nu Opal and Delta Opal (Grigolli et al. 2012). Thus, the seven genotypes tested here did not exert preference in terms of attraction and feeding for boll weevil adults. Oviposition behaviour was not studied but also seems to vary between cotton genotypes. Laboratory assays showed that gossypol is an oviposition stimulant for female boll weevils (Hedin and McCarty 1990), however, some studies revealed that high-gossypol genotypes were less attractive for oviposition (Singh and Weaves 1972; Lambert et al. 1980), while others showed a preference for high-gossypol and high-monosaccharide genotypes (Heidin and McCarty 1995).

Besides the production of secondary metabolites, cotton plants display a series of physical and morphological traits that give resistance against the boll weevil. Red-leaf colour of some cotton genotypes causes antixenotic reactions in adult boll weevils (Isley 1928; Merkel and Meyer 1963). Cotton genotypes displaying the frego bract and okra-leaf traits also have reduced boll weevil damage (Singh and Weaves 1972; Vidal-Neto et al. 2005). None of the genotypes studied here have these physio-morphological resistance traits to the boll weevil. Biological and behavioural aspects, such as those already discussed, must be taken into account during selection for desirable agronomic traits in studies involving plant-insect interactions. Variation in herbivore responses to different types of traits may occur, depending on the degree of host plant specialization (generalist vs. specialist herbivores). The same is true for morphological traits: the frego bract, for example, confers resistance against the

boll weevil but increases cotton susceptibility to stink bugs (Sadras and Felton 2010). Thus, knowing more about herbivores and their host plant interactions is crucial to any further understanding of physical, morphological, or chemical traits that confer resistance to insect pests.

Author contribution

DMM and MCBM conceived and designed research. Material preparation, data collection and analysis were performed by DMM, MCBM, MB, RAL, JCC, and MAB. The first draft of the manuscript was written by DMM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Fig 1 Amounts (mean ± SEM) of total volatile organic compounds (VOCs) emitted from different cotton genotypes. Means with the same letter are not different ($P > 0.05$) by General Linear Model (GLM) and ANODEV and mean comparisons by contrast analyses

Fig 2 Amounts (mean ± SEM) of total monoterpenes (a), sesquiterpenes (b), homoterpenes (c), esters (d), and ketones (e) emitted from different cotton genotypes. Means with the same letter are not different ($P > 0.05$) by General Linear Model (GLM) and ANODEV and mean comparisons by contrast analyses

Fig 3 Proportion (%) of volatile organic compound (VOC) chemical classes emitted from different cotton genotypes

Fig 4 Amounts (mean ± SEM) of total terpenoid aldehydes (TA) from different cotton genotypes in leaves (a), squares (b), and bolls (c). Means with the same letter are not different ($P > 0.05$) by General Linear Model (GLM) and ANODEV and mean comparisons by contrast analyses. As Empire Glandless did not present any TA, it was not included in the analyses

Tables

Table 1 Amounts (mean ± SEM) of volatile organic compounds (VOCs) emitted from different cotton genotypes (ng/24 h)

Table 2 Amounts (mean ± SEM) of terpenoid aldehydes (TA) from different cotton genotypes in leaves, squares and bolls (mg/mL)

592

Figure

Figure 1

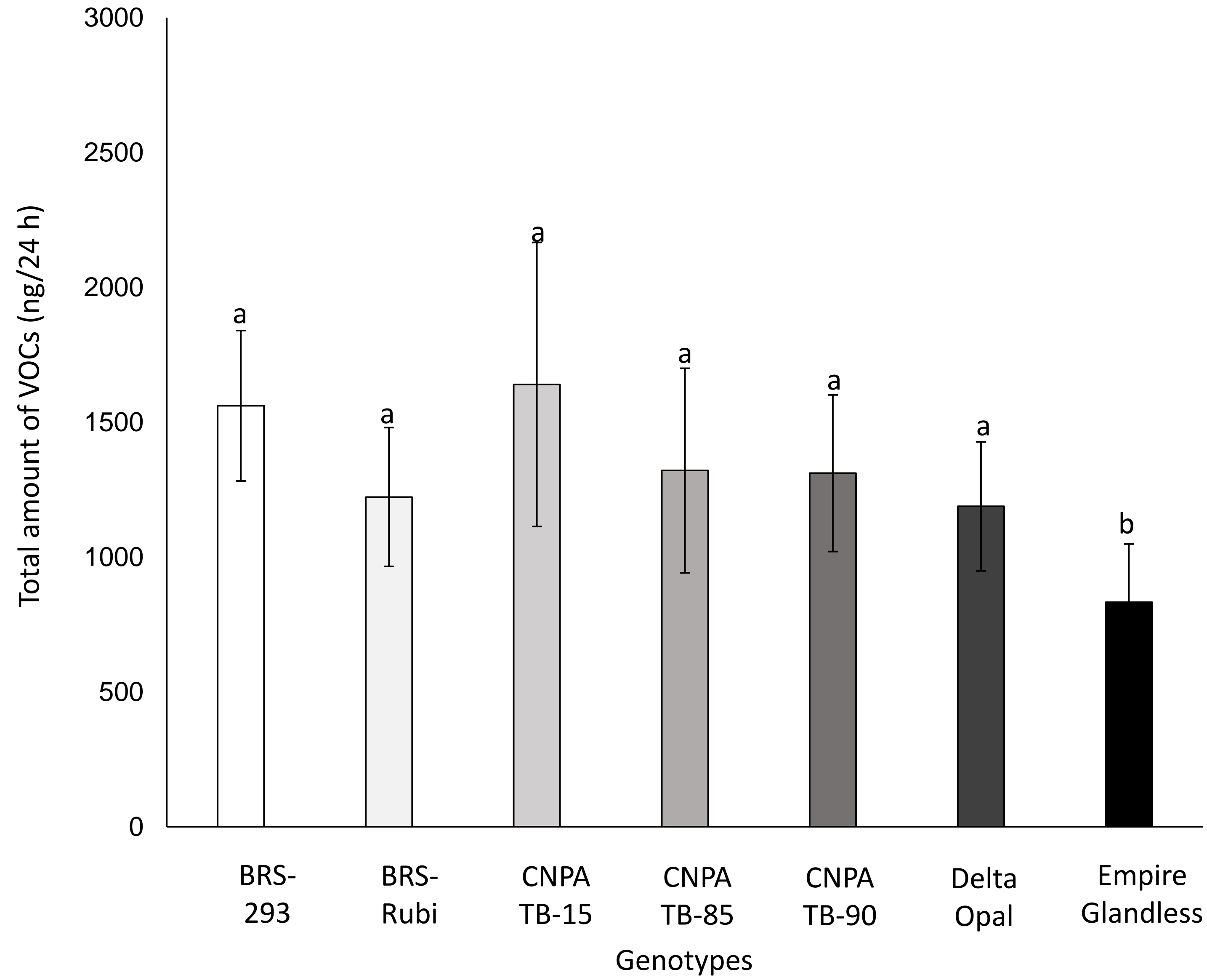
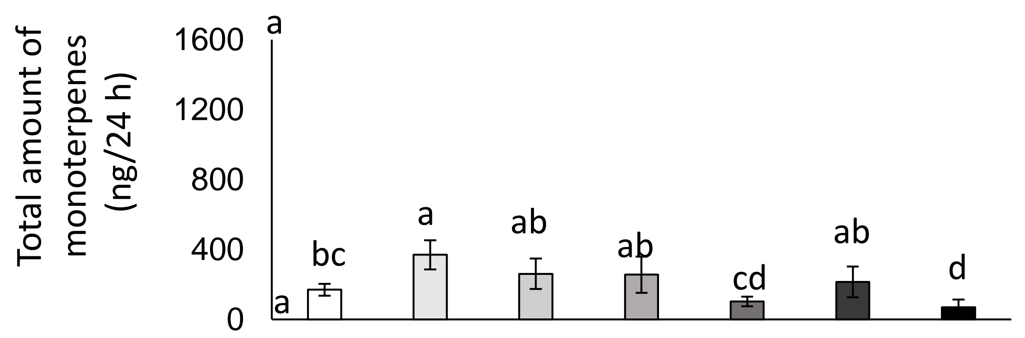
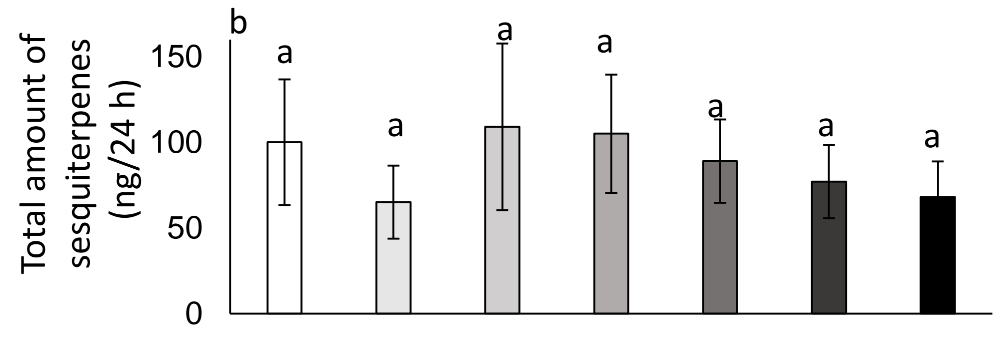
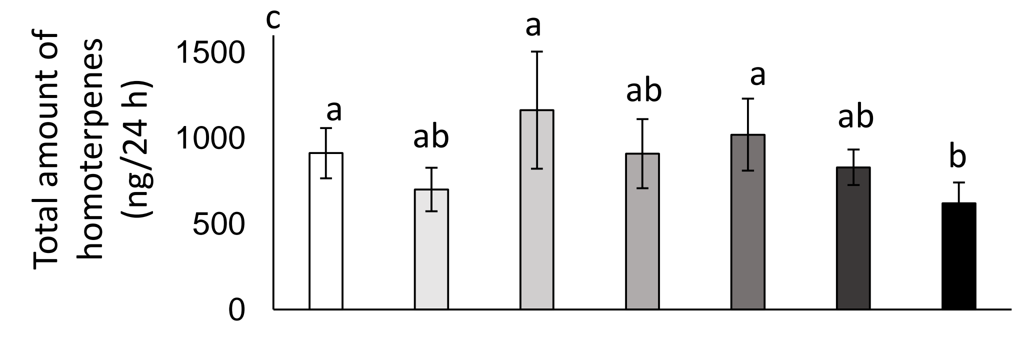
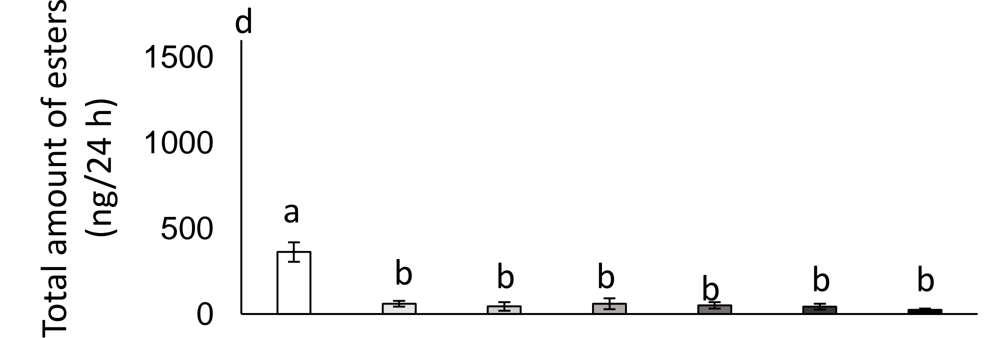


Figure 2









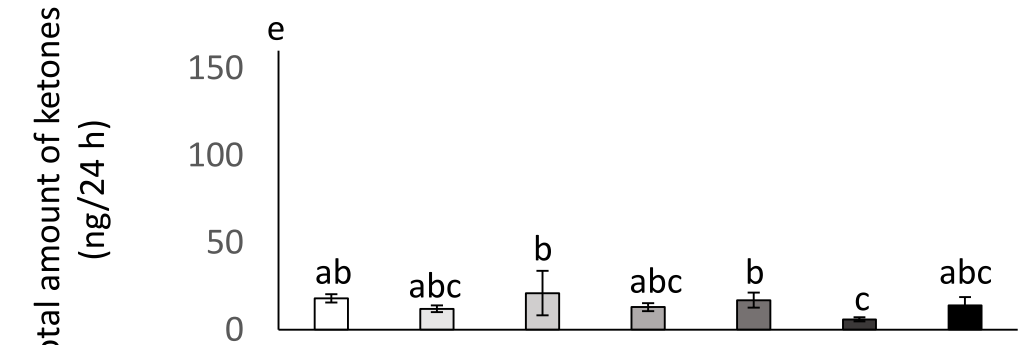


Figure 3

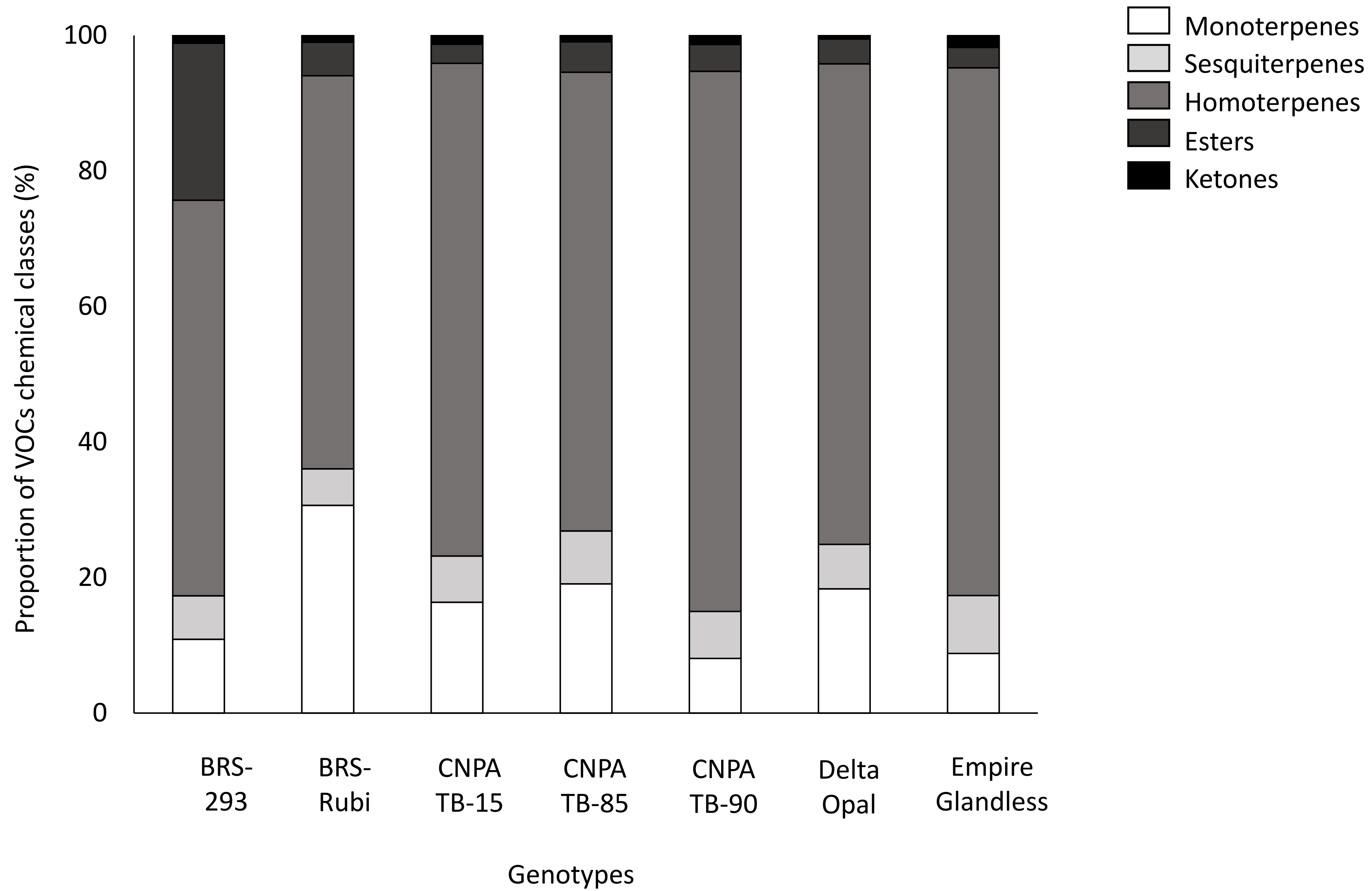
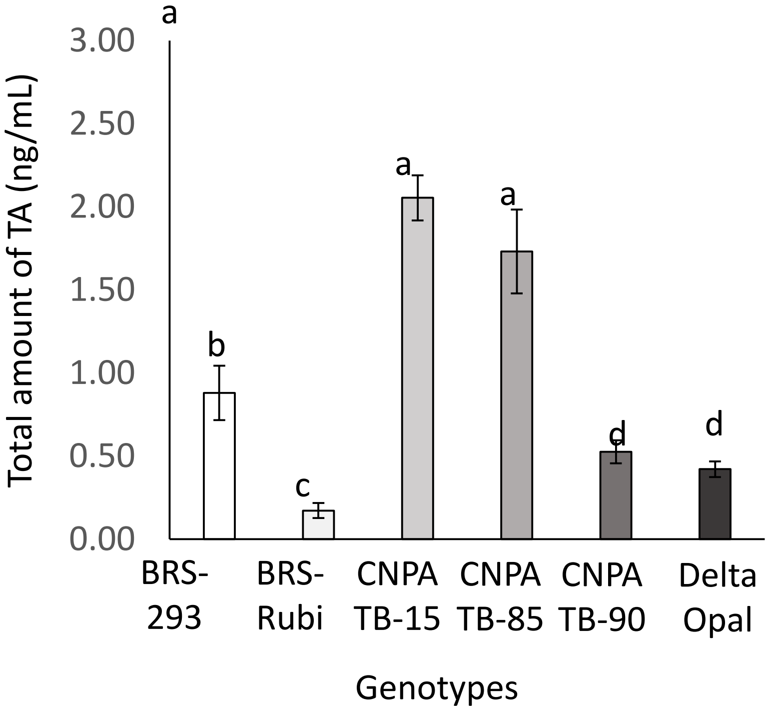
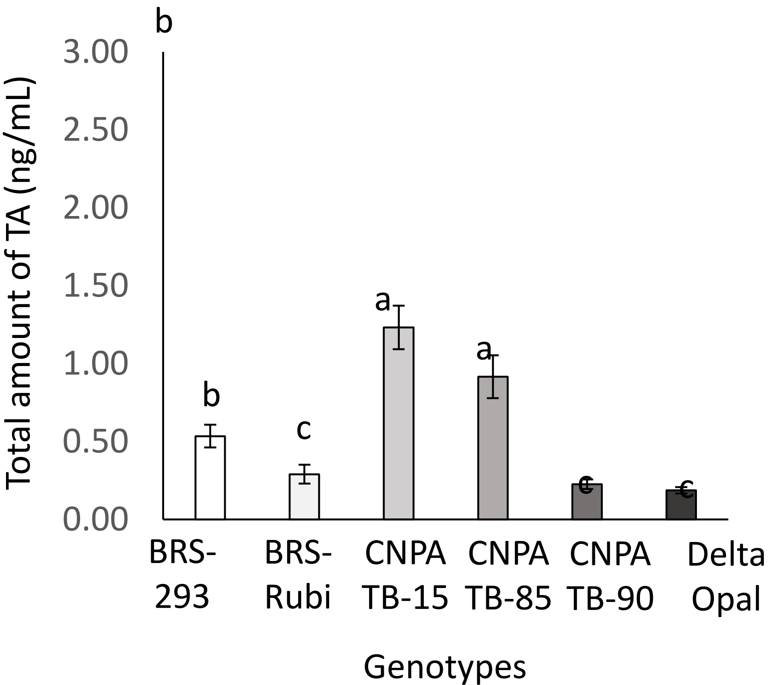
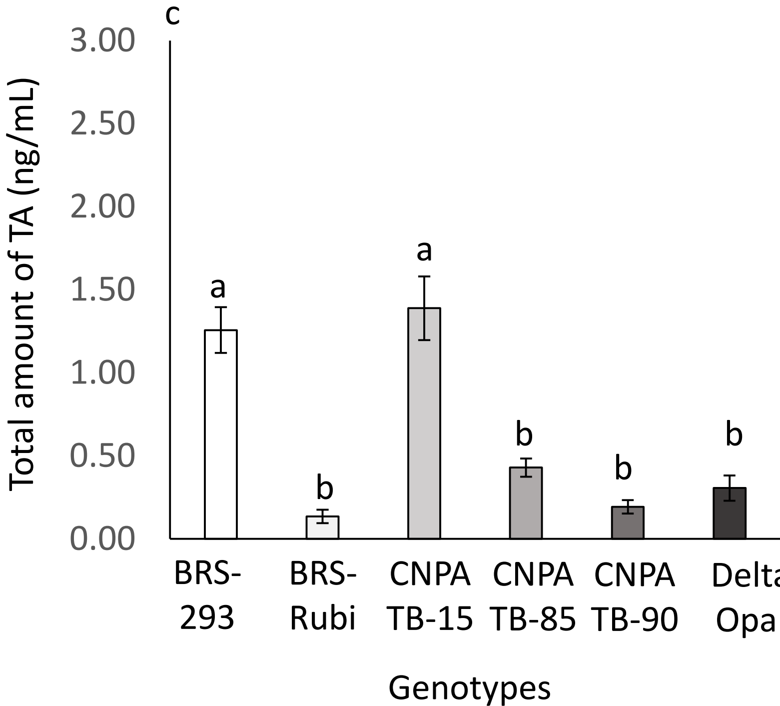


Figure 4







Table

Table 1 Amounts (mean ± SEM) of volatile organic compounds (VOCs) emitted from different cotton genotypes (ng/24 h)

		Genotypes						
Compounds	**RI**	**BRS-293**	**BRS-Rubi**	**CNPA TB-15**	**CNPA TB-85**	**CNPA-TB90**	**Delta Opal**	**Empire Glandless**
α-Pinene	938	41.03 ± 5.90	209.50 ± 47.55	136.90 ± 31.92	185.41 ± 72.09	17.18 ± 4.90	93.11 ± 34.84	0.00 ± 0.00
Camphene	954	7.54 ± 3.35	0.00 ± 0.02	0.00 ± 0.00	5.18 ± 1.74	4.34 ± 0.57	8.22 ± 3.33	0.00 ± 0.00
β-Pinene	981	10.92 ± 2.75	40.57 ± 7.81	17.82 ± 6.50	18.02 ± 4.22	9.98 ± 1.48	17.97 ± 5.97	10.18 ± 2.85
Myrcene	990	23.31 ± 8.76	45.48 ± 12.01	33.58 ± 15.96	34.39 ± 14.84	17.20 ± 2.68	43.19 ± 25.28	0.00 ± 0.00
(*Z*)-3-Hexenyl acetate	1005	37.28 ± 7.17	28.28 ± 10.25	27.92 ± 12.44	16.40 ± 13.96	20.41 ± 8.31	17.36 ± 5.59	17.03 ± 5.32
1-Decyne	1024	0.00 ± 0.02	15.05 ± 6.64	41.20 ± 11.75	18.60 ± 6.94	30.89 ± 4.01	18.47 ± 8.73	35.70 ± 16.91
Limonene	1033	10.95 ± 0.79	20.02 ± 2.36	14.81 ± 7.31	13.83 ± 3.96	13.38 ± 2.78	14.08 ± 2.94	24.97 ± 5.14
(*E*)-Ocimene	1050	62.84 ± 7.70	45.54 ± 10.79	18.34 ± 12.29	14.68 ± 3.74	14.30 ± 7.37	23.19 ± 13.67	20.90 ± 7.37
γ-Terpinene	1063	0.00 ± 0.00	0.00 ± 0.00	9.68 ± 7.95	0.00 ± 0.00	6.32 ± 2.23	0.00 ± 0.00	0.00 ± 0.00
Linalool	1104	13.13 ± 1.14	9.24 ± 1.24	29.70 ± 5.04	5.69 ± 1.29	19.93 ± 6.19	14.70 ± 2.08	14.42 ± 3.95
DMNT[a]	1114	276.06 ± 42.97	174.42 ± 44.25	260.14 ± 66.79	312.47 ± 103.08	258.73 ± 85.90	271.40 ± 53.51	152.64 ± 28.23
Methyl salicylate	1193	324.33 ± 50.31	31.95 ± 6.69	17.06 ± 12.63	64.57 ± 17.23	30.82 ± 10.35	25.37 ± 10.97	6.91 ± 3.21
(*E*)-Caryophyllene	1424	7.70 ± 2.42	24.97 ± 9.18	19.88 ± 14.28	24.27 ± 9.05	44.41 ± 8.93	13.57 ± 2.63	11.82 ± 3.42
Geranylacetone	1449	17.72 ± 2.42	11.55 ± 1.94	21.12 ± 12.75	11.95 ± 2.24	17.29 ± 4.36	5.91 ± 1.21	13.78 ± 4.66
α-Humulene	1461	28.76 ± 0.78	24.81 ± 3.37	47.66 ± 14.22	22.03 ± 10.46	22.43 ± 3.78	19.38 ± 5.10	23.23 ± 10.08
δ-Guaiene		14.35 ± 10.16	7.66 ± 2.46	22.14 ± 8.18	9.01 ± 3.73	8.08 ± 1.79	15.49 ± 4.85	6.48 ± 1.11

	1404																					
δ-Cadinene	1520	11.09	±	3.24	7.89	±	4.26	9.47	±	8.43	47.19	±	11.30	14.07	±	5.39	14.18	±	7.06	11.71	±	0.92
Nerolidol	1556	37.86	±	20.06	0.00	±	0.07	10.35	±	3.57	0.00	±	0.00	0.00	±	0.00	14.61	±	1.76	14.76	±	5.20
TMTT[b]	1575	635.84	±	104.06	525.68	±	82.58	902.32	±	274.62	516.94	±	97.73	760.91	±	124.41	557.73	±	49.90	467.69	±	93.28

[a] (*E*)-4,8-Dimethyl-1,3,7-nonatriene
[b] (*E*-*E*)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene
RI: Retention index

Table 2 Amounts (mean ± SEM) of terpenoid aldehydes (TA) from different cotton genotypes in leaves, squares and bolls (mg/mL)

Compounds	Genotypes						
	BRS-293	BRS-Rubi	CNPA TB-15	CNPA TB-85	CNPA TB-90	Delta Opal	Empire Glandless
Leaves							
Hemigossypolene	0.113 ± 0.030	0.056 ± 0.008	0.797 ± 0.037	0.750 ± 0.132	0.191 ± 0.015	0.116 ± 0.021	0.000 ± 0.000
Gossypol	0.012 ± 0.004	0.002 ± 0.001	0.044 ± 0.007	0.042 ± 0.007	0.017 ± 0.003	0.005 ± 0.002	0.000 ± 0.000
Heliocide H4	0.050 ± 0.011	0.021 ± 0.008	0.183 ± 0.021	0.091 ± 0.017	0.034 ± 0.008	0.004 ± 0.002	0.000 ± 0.000
Heliocide H1	0.270 ± 0.064	0.046 ± 0.018	0.410 ± 0.043	0.321 ± 0.051	0.093 ± 0.018	0.116 ± 0.006	0.000 ± 0.000
Heliocide H3	0.177 ± 0.021	0.013 ± 0.003	0.160 ± 0.007	0.142 ± 0.010	0.050 ± 0.008	0.046 ± 0.005	0.000 ± 0.000
Heliocide H2	0.319 ± 0.034	0.035 ± 0.008	0.461 ± 0.021	0.385 ± 0.034	0.141 ± 0.018	0.135 ± 0.012	0.000 ± 0.000
Squares							
Hemigossypolene	0.118 ± 0.016	0.070 ± 0.021	0.315 ± 0.028	0.246 ± 0.030	0.078 ± 0.011	0.040 ± 0.007	0.000 ± 0.000
Gossypol	0.088 ± 0.009	0.063 ± 0.009	0.067 ± 0.012	0.146 ± 0.031	0.051 ± 0.005	0.077 ± 0.006	0.000 ± 0.000
Heliocide H4	0.025 ± 0.004	0.024 ± 0.006	0.111 ± 0.021	0.052 ± 0.010	0.016 ± 0.003	0.000 ± 0.000	0.000 ± 0.000
Heliocide H1	0.144 ± 0.017	0.084 ± 0.015	0.309 ± 0.042	0.221 ± 0.032	0.022 ± 0.004	0.023 ± 0.004	0.000 ± 0.000
Heliocide H3	0.045 ± 0.010	0.013 ± 0.003	0.109 ± 0.009	0.063 ± 0.009	0.021 ± 0.004	0.012 ± 0.001	0.000 ± 0.000
Heliocide H2	0.115 ± 0.018	0.037 ± 0.006	0.321 ± 0.028	0.188 ± 0.026	0.039 ± 0.004	0.035 ± 0.002	0.000 ± 0.000
Bolls							
Hemigossypolene	0.439 ± 0.056	0.030 ± 0.006	0.329 ± 0.064	0.021 ± 0.002	0.062 ± 0.012	0.048 ± 0.009	0.000 ± 0.000
Gossypol	0.068 ± 0.008	0.001 ± 0.004	0.456 ± 0.012	0.011 ± 0.002	0.001 ± 0.005	0.005 ± 0.001	0.000 ± 0.000
Heliocide H4	0.241 ± 0.024	0.019 ± 0.003	0.173 ± 0.023	0.047 ± .008	0.020 ± 0.003	0.007 ± 0.004	0.000 ± 0.000
Heliocide H1	0.352 ± 0.034	0.058 ± 0.010	0.592 ± 0.063	0.306 ± 0.037	0.069 ± 0.012	0.204 ± 0.056	0.000 ± 0.000
Heliocide H3	0.038 ± 0.004	0.018 ± 0.017	0.048 ± 0.008	0.000 ± 0.000	0.026 ± 0.006	0.002 ± 0.000	0.000 ± 0.000
Heliocide H2	0.120 ± 0.012	0.009 ± 0.001	0.190 ± 0.021	0.045 ± 0.005	0.015 ± 0.002	0.039 ± 0.006	0.000 ± 0.000

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