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Exploitation of herbivore-induced cotton volatiles by the parasitic wasp *Bracon vulgaris* reveals a dominant chemotactic effect of terpenoids

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Abstract Plants emit a wide array of complex blends of volatile organic compounds that can be involved in plant communication with herbivores and their natural enemies. *Bracon vulgaris* Ashmead (Hymenoptera: Braconidae) is a gregarious larval ectoparasitoid that attacks the boll weevil larvae, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), an important pest in cotton plantations in Brazil. This parasitoid species has been studied as a potential biological control agent of *A. grandis*. However, little is known about *B. vulgaris* host foraging behaviour. We have previously demonstrated that female wasps respond to host-associated cues (boll weevil's aggregation pheromone) and host habitat odours, such as cotton volatiles induced by the presence of the boll weevil's pheromone. In the current study, we evaluated the electrophysiological and behavioural responses of *B. vulgaris* to constitutive and herbivore-induced plant volatiles (HIPVs) emitted by *A. grandis*-infested cotton plants at different phenological stages. The results demonstrated that *B. vulgaris* recognizes and responds to reproductive cotton HIPVs and that polar compounds might not be essential for its attraction. Electroantennogram (EAG) recordings and bioassays suggested that the compounds β-myrcene, (*E*)-ocimene, (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT), (*E*)-(1*R*,9*S*)-caryophyllene, and

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(*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), as well as other minor components of cotton blend, can be used by *B. vulgaris* wasps in its host foraging behaviour. Our results show an important role of terpenoids in cotton indirect defence, which is discussed relative to the role of other minor plant volatiles.

Keywords Plant volatiles · Tritrophic interactions · Braconidae · Herbivory · Indirect defence

Introduction

As a consequence of their interactions with biotic and abiotic factors, plants emit a wide array of complex blends of volatile organic compounds which have multiple ecological functions (Pierik et al. 2014). In response to herbivory, plants release specific volatile blends (hereafter herbivore-induced plant volatiles, HIPVs) that are used as cues by natural enemies foraging for prey or hosts (Dicke 1994; Blassioli-Moraes et al. 2016; Boncan et al. 2020). HIPVs are easily detectable and more reliable cues than constitutive volatiles for foraging parasitoids and predators, enhancing their attack against herbivores (De Moraes et al. 2000; Turlings and Erb 2018). For several systems, the attraction of parasitoids to HIPVs has been well documented. However, due to their complex and dynamic nature, only a few of these volatile compounds involved in the attraction of the natural enemies have been identified so far (Dicke and Lucas-Barbosa 2020). Another difficulty in identifying HIPVs attractive components is the capacity of parasitoids to learn to respond to volatile blends and our lack of information on insect's sensory processing and perception of olfactory information (Clavijo-McCormick et al. 2012; Smid and Vet 2016).

Plants produce a broad range of volatile blends of up to hundreds of compounds, including mainly terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives (green leaf volatiles, GLVs), and amino acid derivatives (Dudareva et al. 2013). Behavioural bioassays, and coupled gas chromatography and electrophysiology techniques can be used to identify active compounds from these complex natural blends. Studies employing such techniques have shown that HIPVs are perceived as a collection of components rather than as individual attractants (Bruce and Pickett 2011; van Wijk et al. 2011). The potential use of semiochemical-based strategies to manipulate natural enemies' behaviour has opened new perspectives for pest management strategies (Khan et al. 2008; Pickett and Khan 2016). Thus, research on HIPVs can contribute to the development of environmentally sustainable pest management in an agricultural context with the identification of volatile compounds that mediate tritrophic interactions and their application for crop protection (Blassioli-Moraes et al. 2013).

Bracon vulgaris Ashmead (Hymenoptera: Braconidae) is a gregarious larval ectoparasitoid that attacks the boll weevil larvae, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), and the caterpillar pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidopetera: Gelechiidae), both important pests in cotton plantations in Brazil (Toscano and Carvalho 2000a, b). This parasitoid has been studied as a potential biological control agent of *A. grandis* (Toscano and Carvalho 2000a, b; Carvalho et al. 2002). However, little is known about the foraging behaviour of *B. vulgaris*. We have previously demonstrated that female wasps respond to host-associated cues (boll weevil's aggregation pheromone) and host habitat odours, such as cotton volatiles induced by the presence of the boll weevil's pheromone (Magalhães et al. 2019). Once in the plant, female wasps search for the exact location of *A. grandis* larvae within cotton squares using sensory structures in the antennae and ovipositor (Alves et al. 2014), probably making use of non-volatile substances (Vinson 1976).

In the current study, we evaluated the electrophysiological and behavioural responses of *B. vulgaris* to constitutive and herbivore-induced cotton volatiles emitted by *A. grandis*-infested plants at different phenological stages. As a first step to identify the key compounds used as foraging cues by *B. vulgaris*, we also examined the attractiveness of cotton volatiles fractionated in a silica gel column.

Materials and methods

Insect rearing

Anthonomus grandis was 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 a L:D 14:10 photoperiod). The parasitic wasp *B. vulgaris* was obtained from a laboratory colony raised on third instar *A. grandis* larvae (modified from Wanderley and Ramalho 1996). The wasps were maintained under controlled conditions (25 ± 1 °C, 60 ± 10% RH, and a L:D 14:10 photoperiod) in plastic containers (5.0 l) with a voile fabric lid for ventilation. Cotton plugs soaked in water and droplets of honey were offered to the wasps as moisture and food, respectively, and were renewed three times per week. Following emergence, males and females were kept together for copulation. Five-day-old mated females were used in the experiments because a greater parasitism rate was previously reported for females at this age (Alves et al. 2015).

Plants

Cotton (var. Delta Opal) was 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) and organic substrate (Bioplant Soil Conditioner, MG, Brazil) in a growth chamber under controlled conditions (26 ± 1 °C, 60 ± 10% RH, and a L:D 12:12 photoperiod). Cotton plants used in the experiments were six-week-old at the vegetative stage (up to six expanded true leaves and ca. 30 cm high) and 12-week-old at the reproductive stage (presence of squares).

Dynamic headspace collection

Cotton plants were randomly assigned to the following treatments: undamaged (UD) or boll weevil-damaged (BWD) plants. Herbivore-damaged plants were infested with two virgin adult female boll weevils. Volatile organic compounds were collected from UD (N = 4) and BWD (N = 4) at both vegetative and reproductive stages. Plants were individually placed into glass chambers (internal volume 10.0 l) and their volatiles were collected for 24 h. 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 min^{-1} and drawn out at 0.6 l min^{-1} through an adsorbent tube, Porapak Q (60 mg, 80–100 mesh, Supelco, PA, USA), connected to the system via polytetrafluoroethylene (PTFE) tubing. The difference in flow created a slight positive pressure to ensure that unfiltered air did not enter the system. After 24 h of volatile collection, the adsorbent tubes were eluted with 0.5 ml of redistilled hexane and the headspace samples were concentrated to 100 µl under N_2 flow. Samples were stored in vials at − 20 °C until use in experiments.

Chemical analysis

Volatiles were analysed on an Agilent 7890-A gas-chromatograph (GC) equipped with a flame ionization detector (FID) and a non-polar DB-5MS column (60 m × 0.32 mm id, 0.25 µm film thickness, Supelco). 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 (in distilled hexane) was added to the samples. A 1 µl aliquot of each sample was injected on a splitless injector, with helium as the carrier gas. Data were collected with GC OpenLab.

Identifications were performed on an Agilent 5975-MSD quadrupole mass spectrometer (MS) coupled to a GC (Agilent 7890A) equipped with a DB-5MS column (30 m × 0.25 mm id, 0.25 µm film, Supelco), 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 (RIs), and were confirmed by co-injection of the headspace samples with authentic standards. The RIs were calculated by comparison to the retention times of a series of linear hydrocarbon alkanes (C8–C24) analysed with the same separation method.

Chemicals

Hexane for HPLC (≥ 97% redistilled), α-pinene (98%), camphene 90%, β-pinene (99%), (1*R*,5*R*)-β-pinene (99%), (1*S*,5*S*)-β-pinene (99%), β-myrcene (90%), (*Z*)-3-hexenyl acetate (98%), (*E*)-ocimene (90%), (*R*)-linalool (97%), and methyl salicylate were purchased from Sigma Aldrich (Steinheim,

Germany). Linalool (mixture of isomers, 96%), α-humulene (96%), β-caryophyllene (80%), and limonene (97%) were purchased from TCI-America (Portland, OR, USA). Geranylacetone (96%) and Nerolidol (mixture of isomers, 98%) were purchased from TCI (Tokyo, Japan). (*E*)-4,8-Dimethylnona-1,3,7-triene (DMNT) (95%) and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) (97%) were provided by Dr Michael A. Birkett (Rothamsted Research, UK).

Coupled gas chromatography-electroantennographic detection (GC-EAD)

Electroantennogram recordings were achieved on a Perkin Elmer Autosystem XL GC equipped with a FID, a non-polar DB-5 column (30 m×0.25 mm id, 0.25 µm film thickness, J & W Scientific), and a splitless injector with helium as the carrier gas (1 ml min^{-1}) coupled to an electroantennography detector (EAD; Syntech, The Netherlands). The temperature program was as follows: 50 °C held for 2 min, followed by an increase to 250 °C at 15 °C min^{-1}, and then maintained at 250 °C for 10 min. An antenna was excised and mounted between stainless steel electrodes with electrically conductive gel (MERCUR®). The extreme tips of the flagellum and scape were cut off with a scalpel to ensure good electrical contact. The electrodes were connected to an auto spike interface box and an AC/DC amplifier IDAC-2 (Syntech, The Netherlands). The antenna preparations were maintained under a continuous humidified airflow (1 l min^{-1}) controlled by a flow controller (CS-55, Syntech, The Netherlands). Only peaks that showed depolarisations and polarisations in 80% of the runs were considered antennal responses. Three coupled runs using volatile samples were undertaken for female *B. vulgaris*. The headspace samples consisted of boll weevil-damaged volatiles from vegetative cotton collected for 96 h because in a previous study no qualitative differences were noticed for vegetative and reproductive cotton volatiles (Magalhães et al. 2012).

Electrophysiological responses of *B. vulgaris* to identified compounds (EAG)

The electrophysiological activity of identified compounds was confirmed using the set-up described above (see coupled GC-EAD). The stimulus delivery system employed a piece of filter paper (1×1 cm^2) in a disposable Pasteur pipette cartridge. The stimuli were delivered over the preparation in a constant 1 l min^{-1} airstream and applied (2 s duration) every 60 s interval. A 10 µl aliquot of standard solutions of each identified compound (at 1 mg ml^{-1} in distilled hexane) was applied to strips of filter paper, with the solvent being allowed to evaporate for 60 s before the strip was placed into the cartridge. The test compounds were alternated with hexane controls to allow for the decline in the EAG response of the preparations with time. A mean of the preceding and following hexane control response was calculated for each test compound. The antenna response was normalized to a 0.1 mV artificial signal and recorded using EAG for Windows 2014 (Syntech, The Netherlands). Antennal responses were measured in mV. Antennae from 18 females were tested.

Silica gel column chromatography

Headspace samples of reproductive cotton volatiles were loaded onto a silica gel column to obtain fractions containing non-polar and polar compounds. A disposable Pasteur pipette cartridge with a small piece of cotton wool and 500 mg of silica gel was conditioned by eluting with 5 ml of hexane, 5 ml of ethyl ether, followed by 5 ml of hexane. A 200 µl aliquot of BWD samples was loaded onto the column, and eluted sequentially with 3 ml 100% hexane (non-polar fraction, NPF) and 3 ml 100% ethyl ether (polar fraction, PF), collecting each aliquot as a separate fraction. The fractions were analysed by GC–MS as described above and used in behavioural assays (see Olfactometer bioassays).

Olfactometer bioassays

Behavioural assays were performed using Y-tube olfactometry to determine the responses of five-day-old mated female *B. vulgaris* to volatiles from UD and BWD cotton. A square acrylic block, with a Y-shaped cavity sandwiched between two glass plates, was used as the bioassay arena. The trunk of the apparatus was 8.0 cm, with each arm measuring 8.0 cm. Filter papers containing 5 µl of the headspace samples (equivalent to the volatiles released by one plant in ca. 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 0.6 l min^{-1} and drawn out at 0.2 l min^{-1}. A single parasitic wasp was introduced at the base of the Y-tube olfactometer and was observed for 10 min, and the first choice (arm of the olfactometer where the females enter for the first time) and residence time (the time spent in an arm) were noted. Each insect was used only once, and the filter paper was replaced after three replicates. *Bracon vulgaris* females were assayed until a total of 30 individuals had responded (moved in the arena and chose one of the two treatment tested). After six repetitions, the Y-tube olfactometer and the side on which the treatment was presented were swapped to avoid any positional bias. The bioassays were carried out in a controlled environment room at 25 ± 1 °C and 60 ± 10% RH, on a white bench under artificial lighting (514 lx), between 9:00 and 16:00 h. The following combinations for vegetative and reproductive cotton volatiles were used: (1) UD vs. hexane, (2) BWD vs. hexane, and (3) UD vs. BWD. We also tested the following combinations of fractions obtained from the headspace samples: (1) NPF vs. hexane, (2) PF vs. hexane, (3) NPF vs. PF, and (4) NPF vs. BWD (reproductive cotton).

Statistical analysis

A Generalized Linear Model (GLM) and analysis of deviance with gamma distribution and inverse link function were used to compare the total amount of released volatiles from treatments at vegetative and reproductive stages. To evaluate the influence of all compounds in separating the treatments, principal component analysis (PCA) was applied to the multivariate data. PCA was performed using a correlation matrix and comparison between groups. The electrophysiological responses of the *B. vulgaris* antennae to each synthetic plant volatiles and their respective control (hexane) were analysed by t-tests. Data analysis of the first choice of *B. vulgaris* was performed by logistic regression (using binomial distribution and logit link function) and Wald's χ^2 test to assess significance to random choices (50% of choices for each arm of the olfactometer). Residence time in treatment and control arms was analysed by the paired t-test. All analyses were carried out using R (v.3.6.2) (R Core Team, 2020), except for the PCA that was performed using Paleontological Statistics Software (PAST version 3.10).

Results

Chemical analysis

Chemical analysis of the headspace samples revealed quantitative but no qualitative differences in the volatile profiles (Fig. 1 and Table 1). Treatments ($\chi^2 = 50.62$, df = 1, $P < 0.001$) and plant phenology ($\chi^2 = 16.04$, df = 1, $P < 0.001$) have a statistically significant effect on volatile production. However, there was no interaction effect between treatments and plant phenology ($\chi^2 = 0.03$, df = 1, $P = 0.86$). BWD plants emitted considerable amounts of induced volatiles, leading to major increases in total volatile emission, compared to UD plants (vegetative: $\chi^2 = 21.06$, df = 1, $P < 0.001$; reproductive: $\chi^2 = 98.33$, df = 1, $P < 0.001$) (Fig. 1). The total amount of volatiles did not differ for UD plants from different phenological stages ($\chi^2 = 0.25$, df = 1, $P = 0.61$). However, BWD plants emitted greater amounts of volatiles at the vegetative stage compared to the reproductive stage ($\chi^2 = 20.20$, df = 1, $P < 0.001$) (Fig. 1). The first two PCA components explained 64% of the total variance. A clear separation of treatments according to cotton phenology was shown in the PCA. BWD plants in both phenological stages emitted higher levels of HIPVs and these treatments are in the positive Y-axis of the PCA bi-plot, as most of the compounds increased

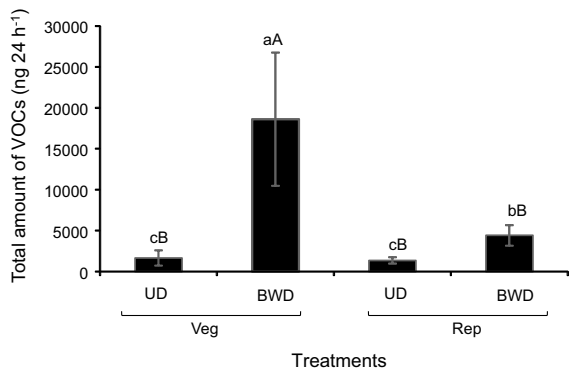

Fig. 1 Amounts (mean ± SE) of total volatiles from undamaged (UD) and boll weevil-damaged (BWD) cotton plants at different phenological stages. Means with the same letter are not different ($P > 0.05$) by General Linear Model (GLM) and analysis of deviance. Lowercase letters are for comparison between treatments (UD and BWD), and uppercase letters are for comparison between phenological stages

Table 1 Amounts (mean ± SE) of individual compounds from undamaged (UD) and boll weevil-damaged (BWD) cotton plants at different phenological stages (ng 24 h^{-1})

#	Compounds	Vegetative		Reproductive	
		UD	BWD	UD	BWD
1	α-Pinene	297.4 ± 173.9	7714.9 ± 3241.4	123.2 ± 74.3	497.8 ± 81.4
2	Camphene	17.7 ± 10.2	97.1 ± 37.2	17.3 ± 4.9	17.0 ± 3.3
3	Benzaldehyde	114.2 ± 76.4	17.5 ± 10.9	79.1 ± 33.9	114.3 ± 19.2
4	(1*S*,5*S*)-β-pinene	102.3 ± 51.7	1382.9 ± 568.7	90.7 ± 19.1	258.6 ± 61.5
5	β-Myrcene	71.9 ± 47.2	2311.6 ± 1296.5	84.7 ± 23.1	306.7 ± 118.3
6	(*Z*)-3-Hexenyl acetate	3.9 ± 3.0	105.7 ± 44.3	23.9 ± 7.9	73.6 ± 20.4
7	(*S*)-Limonene	92.9 ± 72.4	443.3 ± 179.8	26.4 ± 14.6	83.6 ± 39.9
8	(*E*)-Ocimene	11.8 ± 7.7	10.5 ± 8.8	41.3 ± 22.4	285.2 ± 88.6
9	(*R*)-Linalool	92.9 ± 72.4	443.3 ± 179.8	26.4 ± 14.6	83.6 ± 39.9
10	Nonanal	346.8 ± 170.2	431.5 ± 82.8	36.0 ± 7.3	163.4 ± 47.4
11	DMNT	70.0 ± 31.7	2213.4 ± 1358.0	113.9 ± 31.9	724.4 ± 145.2
12	Methyl salicylate	27.4 ± 14.9	206.6 ± 133.3	56.8 ± 15.7	71.0 ± 24.5
13	Indole	46.7 ± 25.2	20.2 ± 8.4	24.3 ± 9.6	65.7 ± 18.6
14	α-Copaene	9.0 ± 4.0	17.0 ± 7.0	19.4 ± 7.1	17.0 ± 6.1
15	(*E*)-(1*R*,9*S*)-Caryophyllene	78.0 ± 47.8	2076.9 ± 534.1	141.7 ± 15.1	513.5 ± 154.8
16	Geranylacetone	104.4 ± 53.1	74.1 ± 20.9	21.3 ± 6.9	60.5 ± 13.7
17	α-Humulene	65.1 ± 27.1	649.1 ± 143.8	79.0 ± 22.2	265.1 ± 110.8
18	δ-Guaiene	7.0 ± 3.3	241.5 ± 278.9	26.1 ± 7.4	39.2 ± 4.6
19	*trans*-Nerolidol	48.4 ± 32.5	50.0 ± 17.7	11.6 ± 7.5	46.4 ± 12.0
20	TMTT	58.5 ± 31.3	28.3 ± 21.6	318.8 ± 58.6	777.3 ± 279.5

The numbers in the first column represent the compounds in Fig. 2

their emission amounts in response to boll weevil herbivory (Fig. 2 and Table 1). Such induction is represented by the vectors' size (long lines) pointing towards BWD treatments in the PCA bi-plot (Fig. 2).

Electrophysiological analysis

Coupled GC-EAD using *B. vulgaris* antenna revealed the presence of nine electrophysiologically active compounds from cotton plants:

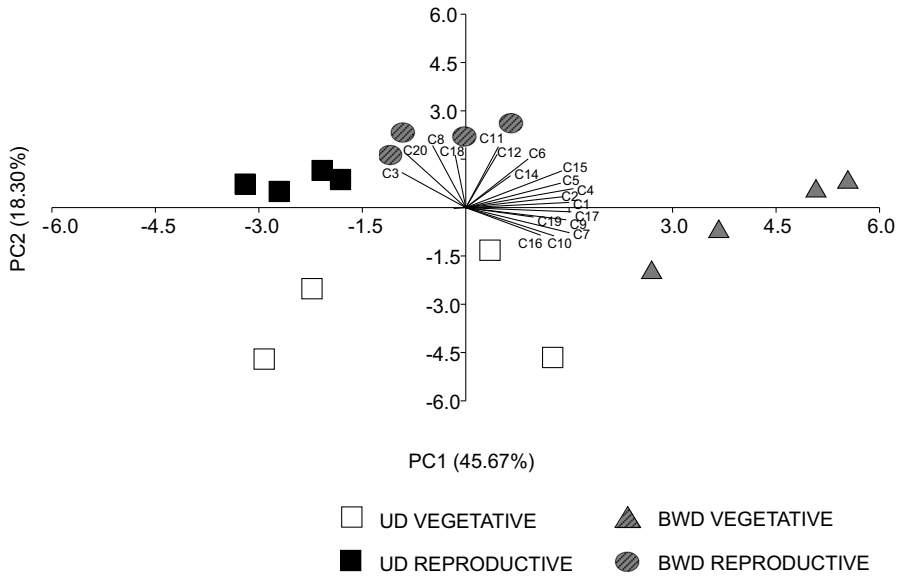

Fig. 2 Principal component analysis (PCA) bi-plot derived from undamaged (UD) and boll weevil-damaged (BWD) cotton plants at vegetative and reproductive stages. Lines represent the vectors (compounds), and their length (relative proportion) the contribution of each compound driving the discrimination among treatments. For compounds identity, see **Table 1**

β-myrcene, (*E*)-ocimene, (*R*)-linalool, DMNT, methyl salicylate, indole, (*E*)-(1*R*,9*S*)-caryophyllene, *trans*-Nerolidol, and TMTT (Fig. 3a). Female antennae elicited statistically significant electrophysiological responses to (*E*)-ocimene ($t=2.19$, $df=34$, $P=0.03$) (*R*)-linalool ($t=4.15$, $df=34$, $P<0.001$), DMNT ($t=3.05$, $df=34$, $P=0.004$), methyl salicylate ($t=2.82$, $df=34$, $P=0.008$), indole ($t=54.95$, $df=34$, $P<0.001$), (*E*)-(1*R*,9*S*)-caryophyllene ($t=2.83$, $df=34$, $P=0.008$), *trans*–Nerolidol ($t=3.17$, $df=34$, $P=0.003$), and TMTT ($t=4.86$, $df=34$, $P<0.001$) identified EAG-active cotton compounds at a stimulus concentration of 1 mg ml^{-1} in relation to hexane, except for β-myrcene ($t=1.78$, $df=34$, $P=0.08$) (Fig. 3b).

Behavioural assays

Females *B. vulgaris* did not show any preference when volatiles from UD cotton (vegetative: $\chi^2=2.08$, $df=1$, $P=0.15$; reproductive: $\chi^2=1.18$, $df=1$, $P=0.28$) were compared to hexane control (Fig. 4a). However, when volatiles from BWD cotton were compared to hexane, they preferred BWD volatiles from reproductive plants ($\chi^2=7.59$, $df=1$, $P=0.006$), but not from vegetative plants ($\chi^2=3.20$,

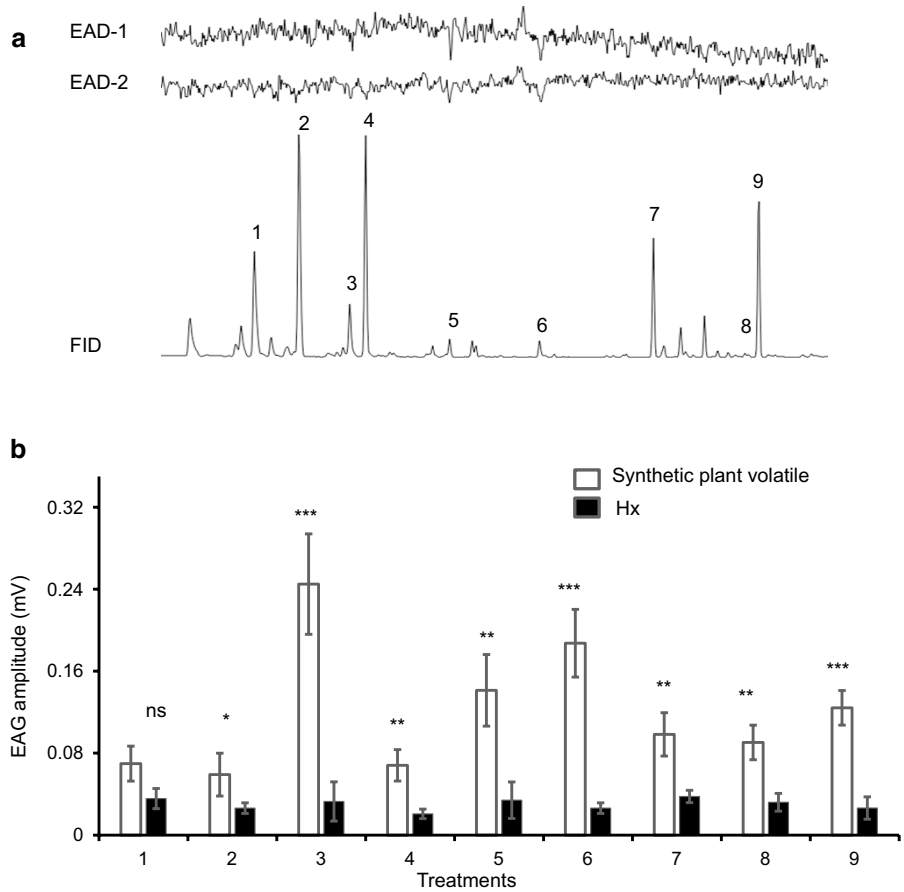

Fig. 3 Representative GC-EAD recordings of *Bracon vulgaris* responses to cotton volatiles (**a**). The upper traces show the electroantennographic response of female antenna (EAD) and the lower trace shows the signal from the FID. The FID peaks marked are those which elicited responses in two or more runs. Electrophysiological responses in mV (mean ± SE) to synthetic compounds identified in cotton plants volatiles by coupled GC-EAD and GC–MS (**b**). Responses were normalized to a 0.1 mV signal. Asterisks indicate differences (* $0.05>P>0.01$, ** $0.01>P>0.001$, and *** $P<0.001$) between the synthetic plant volatiles and HX: hexane. ns: not significant. 1. β-Myrcene, 2. (*E*)-Ocimene, 3. (*R*)-Linalool, 4. (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT), 5. Methyl salicylate, 6. Indole, 7. (*E*)-(1*R*,9*S*)-Caryophyllene, 8. *trans*-Nerolidol, 9. (*E*-*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT)

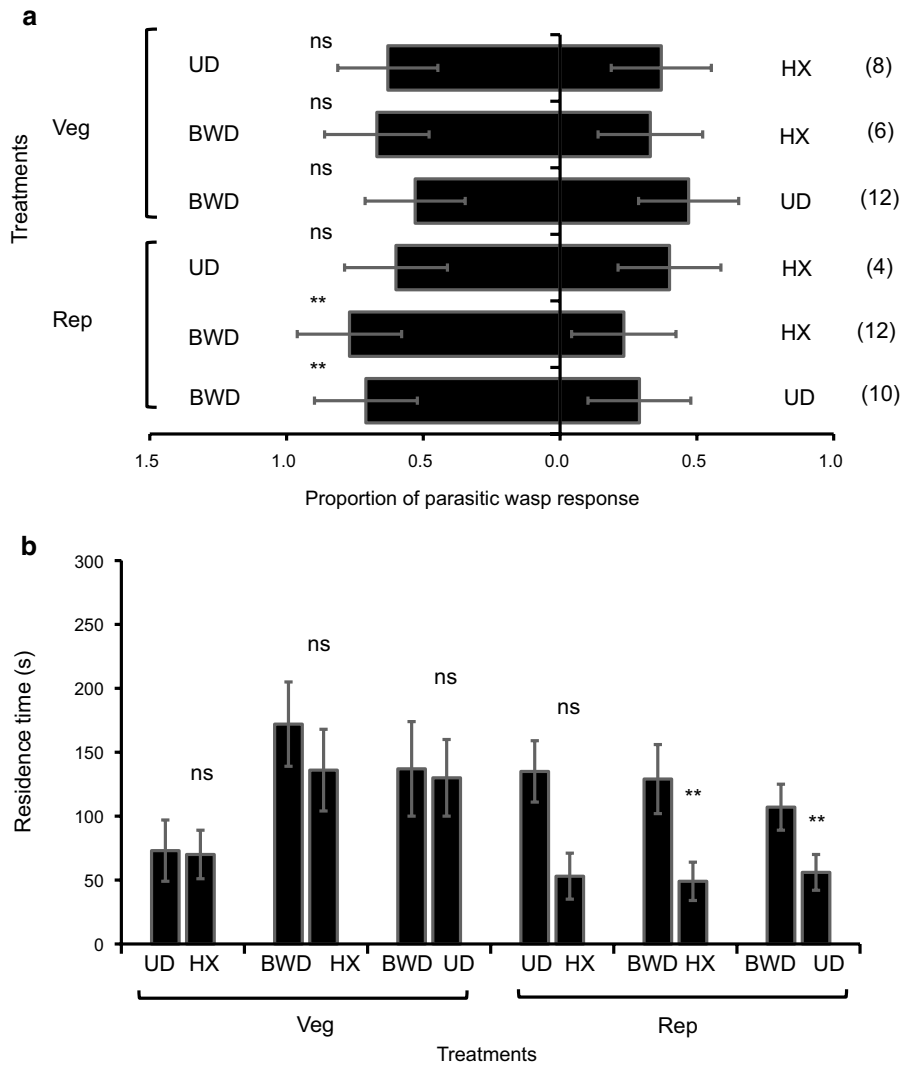

Fig. 4 First choice proportion (**a**) and mean residence time in seconds (**b**) of female *Bracon vulgaris* in a Y-tube olfactometer to volatile organic compounds (VOCs) from undamaged (UD) and boll weevil-damaged (BWD) cotton at different phenological stages. Veg: vegetative stage. Rep: reproductive stage. HX: hexane. Asterisks indicate significant differences (** $0.01 > P > 0.001$) between pairs of treatments. ns: not significant. Error bars indicate 95% confidence intervals calculated from logistic regression parameters in (**a**) and SE in (**b**). Numbers in parentheses indicate *B. vulgaris* that did not respond to both treatments

df = 1, P = 0.07) (Fig. 4a). When volatiles from BWD cotton were compared to UD cotton, females did not show any preference at the vegetative stage ($\chi^2 = 0.13$, df = 1, P = 0.72), but they preferred BWD over UD at the reproductive stage ($\chi^2 = 5.10$, df = 1, P = 0.02) (Fig. 4a). Similar results were obtained for residence time: females did not show any preference when volatiles from UD cotton, at both vegetative (t = 0.14, df = 29, P = 0.89) and reproductive stages (t = 1.95, df = 29, P = 0.06), were compared to hexane, but they spent more time in the arm containing volatiles from BWD cotton at the reproductive stage (t = 2.65, df = 29, P = 0.01) compared to the control arm (Fig. 4b). There was no preference when volatiles from BWD were compared to UD cotton at the vegetative stage (t = − 0.12, df = 29, P = 0.91), but females spent more time in the olfactometer arm containing BWD compared to UD cotton at the reproductive stage (t = 2.49, df = 29, P = 0.02) (Fig. 4b).

We used silica gel column chromatography to separate cotton volatiles into non-polar and polar fractions. The non-polar fraction (NPF) consisted mainly of monoterpenes and sesquiterpenes, while the polar fraction (PF) comprised esters, alcohols, and aldehydes (Fig. 5). When the PF blend was compared to hexane, *B. vulgaris* females did not show any preference ($\chi^2 = 1.18$, df = 1, P = 0.28), but they preferred the NPF blend over hexane ($\chi^2 = 4.52$, df = 1, P = 0.03) (Fig. 6a). Females, however, did not show any preference when PF and NPF blends

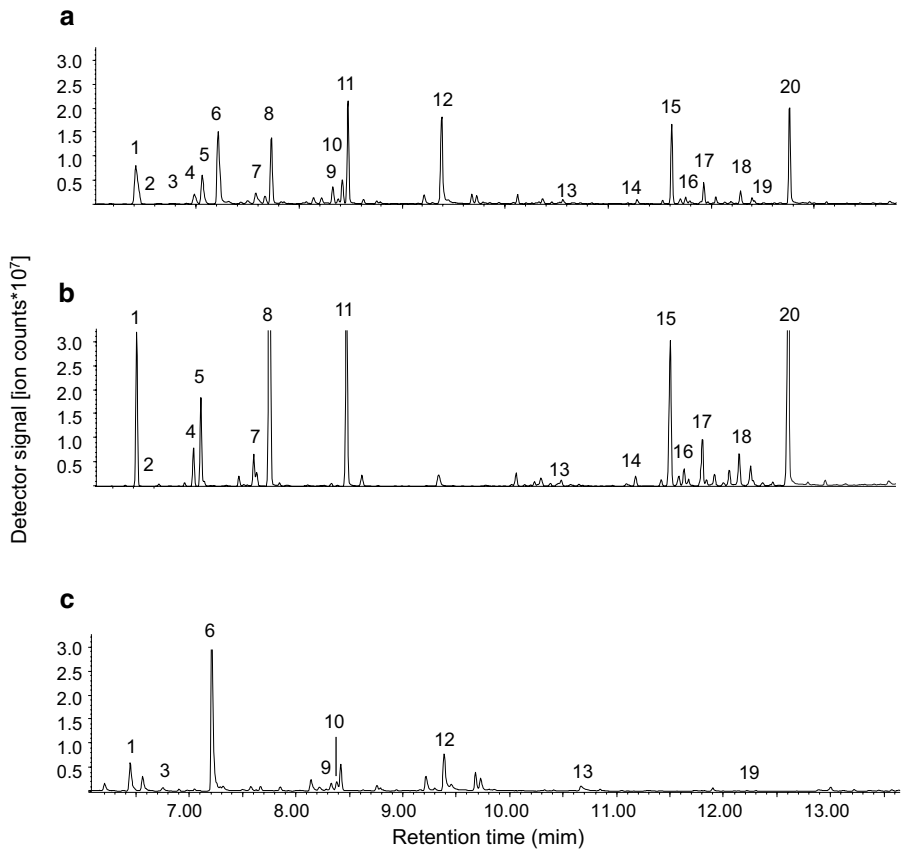

Fig. 5 GC–MS chromatograms of (**a**) natural sample of *Anthonomus grandis*-induced reproductive cotton volatiles, and its silica extracts in (**b**) hexane (non-polar fraction) and (**c**) ethyl ether (polar fraction). The numbers correspond to the compounds identified: 1. α-Pinene, 2. Camphene, 3. Benzaldehyde, 4. (1*S,5S*)-β-Pinene, 5. β-Mycrene, 6. (*Z*)-3-Hexenyl acetate, 7. (*S*)-Limonene, 8. (*E*)-Ocimene, 9. (*R*)-Linalool, 10. Nonanal, 11. (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT), 12. Methyl salicylate, 13. Indole, 14. α-Copaene, 15. (*E*)-(1*R*,9*S*)-Caryophyllene, 16. Geranylacetone, 17. α-Humulene, 18. δ-Guaiene, 19. *trans*-Nerolidol, 20. (*E-E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT)

were compared to each other, with the same number of response (n = 15) to each treatment (Fig. 6a), and when the NPF blend was compared to BWD volatiles ($\chi^2 = 2.08$, df = 1, P = 0.15) (Fig. 6a). The same pattern was found for residence time: females spent more time in the arm of the olfactometer containing the NPF blend compared to hexane control (t = − 2.68, df = 29, P = 0.01), but no significant differences were observed for the other combination of treatments (Fig. 6b).

Discussion

We have demonstrated that the parasitic wasp *B. vulgaris* uses HIPVs as host location cues and cotton phenology seems to play an important role in this process. Similarly, as in a previous study (Magalhães et al. 2012), we found a quantitative variation of volatiles at different cotton growth stages, in which vegetative plants damaged by *A. grandis* had a higher overall volatile release than reproductive plants. No major qualitative differences between the blends from these stages were recorded. Despite the greater amounts of HIPVs emitted by vegetative cotton, female wasps were only attracted to reproductive BWD cotton volatiles. This corresponds well with boll weevil's biology and ecology. Cotton leaves are a resource present throughout plant development and it is known that adults occasionally feed on them (Showler 2008). However, boll weevil adults are found in low densities during cotton vegetative stage (Neves et al. 2018). Their population growth is associated with cotton reproductive phenological stage, as adults feed and oviposit on cotton reproductive organs (squares and bolls), and immatures develop inside those structures. Moreover, Braconidae parasitic wasps are known to search for food on the nectaries at the base of cotton squares (Adams et al. 1969; Pallini et al. 2006). *Bracon vulgaris* preference for reproductive cotton HIPVs agrees with its behaviour parasitizing *A. grandis* larval stages. It is well known

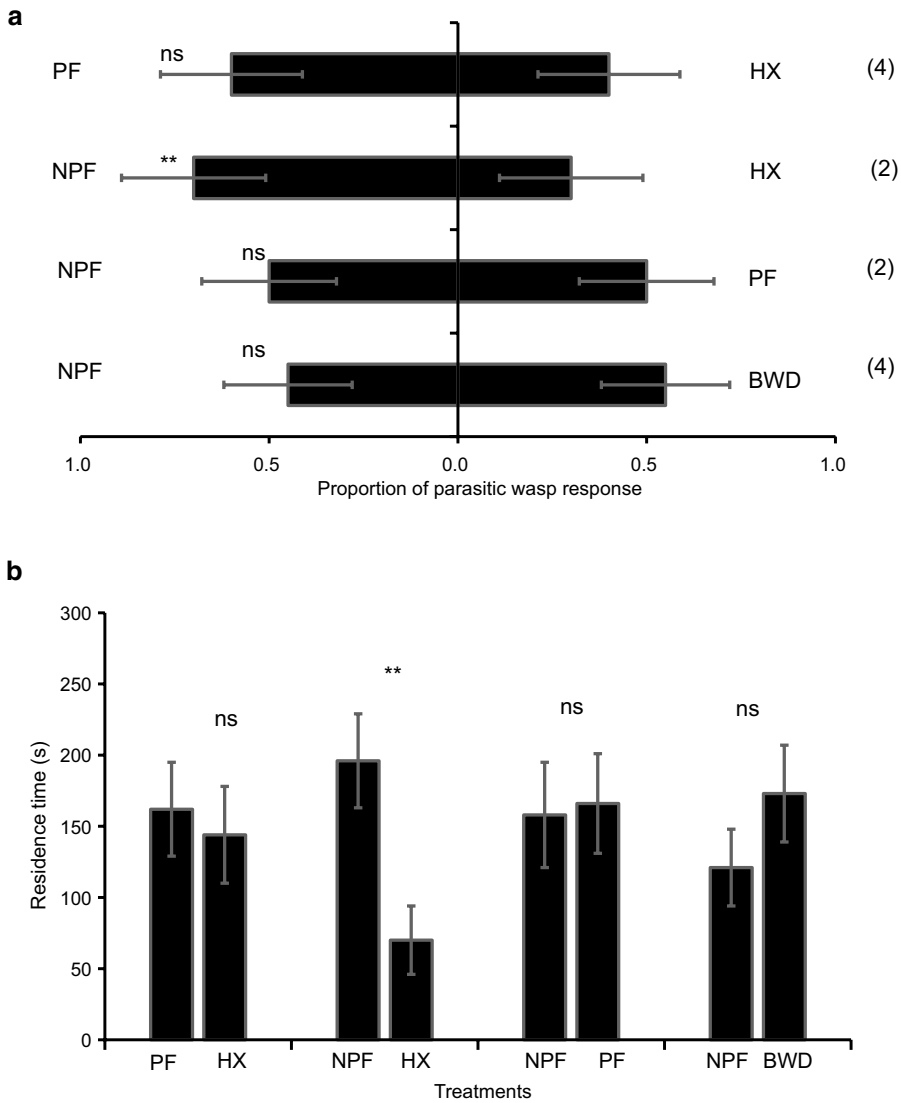

Fig. 6 First choice proportion (**a**) and mean residence time in seconds (**b**) of female *Bracon vulgaris* in a Y-tube olfactometer to synthetic and natural cotton volatile organic compounds (VOCs). BWD: boll weevil-damaged cotton volatiles (natural sample). NPF: synthetic blend comprised of non-polar compounds found in the natural cotton sample. PF: synthetic blend comprised of polar compounds found in the natural cotton sample. HX: hexane. Asterisks indicate significant differences (** $0.01 > P > 0.001$) between pairs of treatments. ns: not significant. Error bars indicate 95% confidence intervals calculated from logistic regression parameters in (**a**) and SE in (**b**). Numbers in parentheses indicate *B. vulgaris* that did not respond to both treatments

that parasitoids use different cues, including several odour sources, to locate their hosts (Rutledge 1996; Colazza et al. 2004; Magalhães et al. 2019). Due to their greater mass area, plants produce higher levels of volatiles compared to eggs or small insects' larvae, and as HIPVs are species-specific, these odours cues are reliable and detectable to parasitoids. Interestingly, *B. vulgaris* seems to use both host-associated (pheromone) and host habitat odours (plant volatiles) as cues for host finding (Magalhães et al. 2019). In addition, because *A. grandis* goes through overlapping generations during the cotton season, induced volatiles (by herbivory or presence of pheromone) and *A. grandis* aggregation pheromone itself might be reliable cues for *B. vulgaris* to find larvae hosts. Further studies should evaluate whether *A. grandis* larvae inside cotton bolls also emit HIPVs and their effect on conspecifics adults and *B. vulgaris* wasps.

Terpenoids are the major class among cotton volatiles (Optiz et al. 2008) and we found out that they comprised 70% of the total blend composition. Increased levels of terpenoid emission were observed in response to feeding by *A. grandis* compared to undamaged plants suggesting that they might be involved in defence against herbivores. The attraction of parasitoids and predators to terpenoids emitted by plants infested with their host/prey has been extensively shown in the literature (Dicke

1994; Blassioli-Moraes et al. 2016; Turlings and Erb 2018; Boncan et al. 2020). Indeed, in this study, EAG recordings from *B. vulgaris* showed that six of nine active compounds were terpenoids ((*E*)-ocimene, (*R*)-linalool, DMNT, (*E*)-(1*R*,9*S*)-caryophyllene, *trans*-Nerolidol, and TMTT). All EAG active compounds (including indole and methyl salicylate) have previously been described as attractive to other parasitoid species (see e.g., Blassioli-Moraes et al. 2016; Turlings and Erb 2018). These compounds were not exclusively released from reproductive cotton. Thus, their proportion might be an important cue for host location. Y-tube bioassays were carried out to determine the behavioural role of the identified EAG active compounds individually and as a blend, but they did not elicit any behavioural response on female wasps (data not shown). The above-mentioned compounds were not attractive at the natural concentration and ratio emitted by herbivore-damaged cotton at the reproductive stage. Usually, compounds that are not individually attractive can become attractive when they are together in a blend (Bruce and Pickett 2011; van Wijk et al. 2011; Magalhães et al. 2018). However, this was not the case and maybe compounds present in minor amounts in the natural blend could play an important role in full attraction of *B. vulgaris* (Clavijo-McCormick et al. 2014). Moreover, different cotton genotypes usually emit volatile blends with different concentrations and ratios among the compounds (Magalhães et al. 2016, 2020), suggesting that *B. vulgaris* might have olfactory plasticity in the use of habitat odours for host finding. Therefore, further studies should focus on the optimization of synthetic blends and the potential role of minor components to establish the most effective composition.

In order to determine the most attractive compounds in the HIPVs natural blend, different solvents were used to separate the non-polar (or less polar) from the polar compounds using a silica gel column. The hexane extract (non-polar fraction, NPF) was attractive to the parasitic wasps when tested against hexane control, whereas the ethyl ether extract (polar fraction, PF) was not attractive to the wasps. Most of the EAD active compounds were present in the NPF, i.e., β-myrcene, (*E*)-ocimene, DMNT, (*E*)-(1*R*,9*S*)-caryophyllene, and TMTT, while (*R*)-linalool, methyl salicylate, indole, and *trans*-Nerolidol were present in the PF. Although there was no significant difference in the attraction of the parasitic wasp when the natural HIPVs blend was tested against NPF, the latter was not more attractive than the PF. Overall, our experiment suggests that highly polar compounds do not seem to be essential for host location by *B. vulgaris*, but the exact compounds mediating its attraction remain to be determined. Another Braconidae species, *Microplitis rufiventris* Kokujev, showed a similar behaviour when *Spodoptera littoralis* (Boisduval)-induced maize volatiles were filtered on a silica gel column: female wasps were highly attracted to a blend from which polar compounds had been removed (D'Alessandro and Turlings 2005). On the other hand, a reduction of a few polar compounds strongly affected *Cotesia marginiventris* (Cresson) attraction (D'Alessandro and Turlings 2005), demonstrating that natural enemies' attraction requires the presence of specific blends of volatiles.

Plant volatiles have an important effect on the searching behaviour of natural enemies. Due to their complexity, the identification of the biologically relevant compounds for host location is not an easy task, but fundamental for the improvement of biological control strategies. Our results show that *B. vulgaris* has an innate response to cotton HIPVs. However, we cannot ignore that, in field conditions, its response can be enhanced by learning the association of different cues, such as HIPVs emissions and pheromones, as herbivore enemies can use naïve and learned responses regardless of their dietary specialization (Steidle and Loon 2003).

Further experiments should focus on the identification of minor compounds, as these molecules could convey important information for natural enemies regarding the identity, quantity, and properties of their prey/hosts (Clavijo-McCormick et al. 2012). Another challenge is that plants can emit several different compounds comprising a great variety of isomeric forms which can restrict the use of synthetic standards if they are not a 100% pure. Previous studies have demonstrated that natural enemies can distinguish different isomeric forms of volatile compounds (Allmann and Baldwin 2010; Roh et al. 2017). In summary, the current study demonstrated that *B. vulgaris* is attracted to reproductive cotton HIPVs and that highly polar compounds might not be essential for its attraction. These results suggest that the non-polar EAG active compounds, as well as other minor components of cotton blend, can be used by *B. vulgaris* wasps in its host foraging behaviour. The

indirect plant defence based on HIPVs has a great potential to be applied in integrated pest management programs in different systems, including cotton crops, where braconid wasps can find both food and hosts. The selection of cotton cultivars that naturally emit attractive HIPVs or the use of slow releaser dispensers loaded with synthetic HIPVs can greatly enhance the presence of parasitoids in the field.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate All authors declared that no competing interests exist. All authors and relevant institutions have read the submitted version of the manuscript and approve its submission.

Consent for publication The work as submitted has not been published or accepted for publication, nor is being considered for publication elsewhere, either in whole or substantial part.

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Izabela Thaís Fidelis Alves Da Silva This research is part of a PhD project of Izabela T.F.A. da Silva devoted to understand the role of cotton HIPVs emitted in response to *Anthonomus grandis* herbivory on the foraging behaviour of *Bracon vulgaris*.

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