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Response of *Trichogramma pretiosum* females (Hymenoptera: Trichogrammatidae) to herbivore-induced Bt maize volatiles

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Abstract

Parasitoids use herbivore-induced plant volatiles (HIPV´s) to locate their hosts. However, little is known about variations in HIPV´s production in genetically modified maize plants that are herbicide tolerant (singular event), insect resistant (Bt plants, singular event), or both herbicide tolerant and insect resistant, like staked events. We investigated the olfactory responses of the egg parasitoid *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) to HIPV´s produced in maize (*Zea mays*) herbicide-tolerant and insect-resistant plants or their stacked events in response to damage caused by *Spodoptera frugiperda* during nighttime and daytime infestations. Real-time reverse-transcription PCR was used to assess whether the presence of one or more *Bacillus thuringiensis* (Bt) proteins and the time of induction of HIPV's affected the expression of genes in plants under herbivore attack. The results showed that compounds were released during both nocturnal and diurnal infestations. However, some HIPV´s were released exclusively in infestations that started during the night by non-Bt plants and they were highly attractive to parasitoids. HIPV´s produced by non-Bt plants were more attractive to parasitoids than those released by Bt plants in infestations that started during the night. However, glyphosate-tolerant maize plants were more attractive to parasitoids than isogenic plants. The expression of the analyzed genes TPS10, TPS23, LOX10, and STC1 was higher in infestations that started during the night. In this study, we discuss the possible causes of the unresponsiveness of *T. pretiosum* females to HIPV´s produced by Bt maize.

Keywords Gmos · Plant volatile compounds · qRT-PCR · Multitrophic interactions · Hipv´s · Biological control

Introduction

After insect herbivore attack, plants activate biochemical pathways that trigger defense responses (Turlings and Tumlinson 1992; De Lange et al. 2016; Mumm and Dicke 2010). These responses include the production and release of herbivore-induced volatile compounds (HIPV´s), which are volatile organic compounds (VOC´s) that can be used by natural enemies to find their host or prey (Dicke et al. 1990a, b; Turlings et al. 2000; Dicke and Baldwin 2010; De Lange et al. 2016).

It is known that plants respond differently to herbivory depending on the time of day when the damage was inflicted (Greenham and McClung 2015). The synthesis of some VOC´s is light dependent (Paré and Tumlinson 1997a, b). Therefore, considering that insect herbivores attack plants at different times of the day, induction responses during the day or at night can modify the pattern of release of VOC´s (Arimura et al. 2008), directly affecting foraging by beneficial insect like predators and parasitoids (Price et al. 1980; De Lange et al. 2016; Naranjo-Guevara et al. 2017).

Some studies show an increase in the foraging of beneficial insects to volatiles induced during the night after herbivory of insect pests. What increases the chances of encounter between predator/parasitoid and prey/host, providing efficiency in pest control in the field (Batool et al. 2014; Naranjo-Guevara et al. 2017). Although most egg parasitoids perform foraging during the day, that is, they are diurnal

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individuals, it is known that most of the chemical tracks used by them are produced during the night and emitted in the early hours of the day (Winkler et al. 2009; Signoretti et al. 2012). For example, compounds induced during the night by adult Lepidoptera females, and released in the early hours of the day are used as chemical clues by daytime parasitoids, such as those of the genus *Trichogramma*.

HIPV´s include terpenoids, aliphatic green leaf volatiles (GLVs), and benzenoids/phenylpropanoids (Dudareva et al. 2006). The temporal pattern of release of HIPV´s depends on the regulation of some genes and the complexity of the chemical compounds, such that complex VOC´s are released later than simpler compounds (Dudareva et al. 2004). The GLVs are released earlier because of their simpler chemical structure (for example, alcohols and acetates, structurally simple compounds). However, GLV's do not provide specific cues to natural enemies about host location or prey (Hilker and Meiners 2006; Tamiru et al. 2011; Naranjo-Guevara et al. 2017). Therefore, like the group of terpenes, which has a more complex chemical structure, the compounds are emitted by plants after the single chain compounds and, therefore, provide more specific and reliable chemical clues about the location of the host. (Hoballah and Turlings 2005; D'auria et al. 2007; Allmann and Baldwin 2010). Generalist insects are expected to respond to VOCs released soon after herbivore damage, such as GLV's, whereas specialized insects, including parasitoids, respond primarily to terpenes (Peñaflor et al. 2011).

Transgenic crops, including those producing *Bacillus thuringiensis* (Bt) insecticide proteins, allow the development of new and efficient integrated pest management strategies and are the main tool for controlling caterpillars in Brazil (Storer et al. 2012), by reducing insecticide applications (Christou et al. 2006; Storer et al. 2012). Several Cry proteins were combined in genetically modified (GM) plants to delay the development of insect resistance to plant chemicals, broaden the target spectrum, and simplify crop management (Head et al. 2017). In addition, plants with multiple traits, such as herbicide tolerance and insect resistance, have become increasingly used (ISAAA 2018). Therefore, hybrid plants with multiple traits, including the presence of one or more Cry proteins and herbicide tolerance, and plants with single traits, including the production of insecticide proteins or herbicide tolerance, are commercially available (ISAAA 2018).

Some studies evaluated the role of Bt genes in the production of HIPV´s by plants and the effect of these compounds on herbivore attack (Anderson and Alborn 1999; Yan et al. 2004; Turlings et al. 2005; Dean and De Moraes 2006; Himanen et al. 2009; Téllez-Rodríguez et al. 2014; Liu et al. 2015; Jiao et al. 2018; Nascimento et al. 2020) and host-seeking behavior of insect herbivores (Dicke et al. 1990a, b; Turlings et al. 1998; De Moraes and Mescher 2004). However, these studies evaluated variations between Bt and isogenic lines plants (Smith et al. 1996; Schuler et al. 1999; Yan et al. 2004), but did not investigate possible variations in emissions in plants with multiple traits.

Beyond that, the induction of VOC´s may be altered in GM crops via unpredictable phenotypic changes in plant defense systems due to pleiotropic effects or insertion of exogenous genes (Schuler et al. 1999), which may interfere with other trophic levels, for example, affecting parasitoid foraging. In addition, few studies have analyzed host-seeking behavior in insect parasitoids attracted to VOC´s induced during the night, considering that volatiles that trigger specific cues, such as terpenes, are released after hours of herbivore damage (Naranjo-Guevara et al. 2017).

Therefore, in view of modifying VOCs emitted after insects herbivore attack in GM plants, this study evaluated whether volatiles released after *Spodoptera frugiperda* (J.E. Smith), 1797 (Lepidoptera: Noctuidae) feeding damage were affected by genetic modifications in maize plants with single and multiple traits and the effect of these compounds on the behavior of the egg parasitoid *Trichogramma pretiosum*. The plant models used were four transgenic maize hybrids, and the isogenic line, with the same genotype but no genetic modifications. The polyphagous herbivore *S. frugiperda* is considered one of the main pests of maize crops and feeds on all stages of plant development (Montezano et al. 2018). The egg parasitoid *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) is a microhymenopteran that parasitizes eggs of several lepidopteran species of agricultural importance.

This study evaluated (i) the effect of time and duration of induction of HIPV´s and *T. pretiosum* olfactory behavioral responses to them; (ii) possible variations in the chemical profile between plants with single or multiple transgenic traits and isogenic plants; and (iii) possible variations in the expression of constitutive genes (LOX10, TPS10, TPS23, and STC1) between the maize hybrids. We analyzed the genes of one lipoxygenase and two terpene synthases, enzymes involved in the production of volatile compounds that attract *S. frugiperda* parasitoids.

Methods and materials

Plants

Seeds of commercial hybrids maize, DKB390 (isogenic line), DKB390 YieldGard VT PRO ™ (Cry1A.105 + Cry2Ab2), DKB390 VT PRO 2™ (Cry1A.105 + Cry2Ab2, and glyphosate herbicide-tolerant), DKB390 VT PRO 3® (Cry1A.105 + Cry2Ab2, Cry3Bb1 and glyphosate-tolerant) and Ag 3700 RR2 (CP4 EPSPS, glyphosate-tolerant), from Dekalb (Monsanto, St. Louis, USA) were planted in

2L-polyethylene pots filled with 1.5 kg of soil. Maize plants used in the bioassays were used 10-12 days after emergence with three fully expanded leaves (V3). At this stage, maize plants were naturally attacked by *S. frugiperda*. Plants were kept in greenhouses (25 ± 5 °C, 70 ± 15% relative humidity (RH), 12:12 light (L):dark (D)) and irrigated as needed.

Insects

Eggs of *S. frugiperda* used in this bioassay were obtained from the laboratory of the Biological Control in Embrapa Maize and Sorghum Research Center. The adults were kept in cages (20 cm in diameter and 30 cm in height) with tissue paper inside the cages for oviposition. Diet solution (25 g sugar; 1 g ascorbic acid; 1 g corn glucose in 1000 mL distilled water) was provided. Eggs are harvested after four days, placed in plastic bags, and stored with controlled temperature (25 ± 1 °C; 70 ± 10% UR, 12 h). After 48 h larvae were transferred and individualized into 50 ml plastic cups with artificial diet. The larvae were kept in the cups until emergence of the adults and then were transferred to the cages to mate (Valicente and Barreto 2003).

The egg parasitoids of *T. pretiosum* were provided by the Koppert® Biological Systems Company and maintained under controlled temperature and relative humidity until the beginning of the experiment. Parasitoids were sexed using a stereomicroscope and the antennal morphology. (Querino and Zucchi 2003). All vials were maintained in incubators (25 ± 1 °C, 60 ± 10% UR, 12–12 h).

Plant treatment

The induction of VOCs was performed by infesting each plant with ten third-instar larvae. Two "clip cages" (3.5 cm in diameter) containing five larvae per cage were coupled in plants. This frame-less clip cage is used to contain small insects on host plants.

Diurnal herbivory (DH, 08:00 a.m. to 02:00 p.m) and nocturnal herbivory (NH, 02:00 a.m. to 08:00 a.m) assays were performed after 6 h of herbivore damage, and 24-h feeding assays were performed after 24 h of herbivore attack (24 h H, 08:00 a.m. to 08:00 a.m of the following day). Larvae were removed from plants before egg parasitoid behavioral tests, VOC analysis and gene expression. Undamaged plants (UD) were used as controls.

Leaf area consumption (LAC)

The extent of damage caused by larval feeding on maize plants was determined by measuring leaf area consumption (LAC). This parameter allows comparing leaf consumption by larvae and plant gene expression. The period of permanence and methodology of plant infestation are described in the item "Plant treatment". The experiments were carried out in a greenhouse. LAC was estimated using an LI-3100C leaf area meter (LI-COR®).

Tissue collection for gene expression analyses

After plant infestation, larvae were removed for plant tissue collection. The area of the leaf that was used to quantify feeding damage measured area corresponded to an upper and lower limit of 2 cm from the site of damage, and the leaf was sectioned transversally. Immediately after collection, the samples were wrapped in aluminum foil, labeled, frozen in liquid nitrogen, and stored at –80 °C until use. Three specimens were collected per sample, and three biological replicates were collected for each treatment.

Real-time PCR

Total RNA was extracted from leaf tissues using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendations. RNA quantification was performed using a NANODROP ND-1000 spectrophotometer. The extracted RNA was stored at –80 °C until use.

cDNA synthesis

cDNA was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and stored at –20 °C until use.

Primers and genes

The sense and antisense primers for each gene are shown in Table 1. The selected target genes were terpene synthase (TPS) 23, TPS10, sesquiterpene cyclase (STC) 1, and lipoxygenase (LOX) 10. Ubiquitin (UBQ) was used as a reference gene. The genes were selected because they are considered key genes involved in plant defense responses to pest insects. Sequences obtained at the National Center for Information in Technology (NCBI, USA http://www.ncbi.nlm.nih.gov/).

RT-qPCR analysis

The reaction efficiency for each target gene was determined using a four-point standard curve and a 1:10 dilution factor. Amplification specificity was evaluated from the melting curve. The qPCR reactions used for validation and expression analysis were performed in a 10-µL reaction volume containing 3.0 µL cDNA (diluted 50X), 5 pmol of each primer, and 1X Fast Master Mix (Applied Biosystems). Reactions were conducted in a 7500 Fast Real-Time PCR System (Applied Biosystems) following the manufacturer's

Table 1 Primer pairs used in RT-qPCR

Gene/accession number	Sense/antisense (5′→3′)*
TPS10/NM_001112380.1	CGTGGTGGATGATACGAAATG/GCGTCTGGTGAAGGTAATGG
TPS23/EU259633.1	TGCTCACGCAGTTGTTTATGA/CATTGCTCCACGCCTTCTT
STC1/NM_001112412.1	GGAGCAGCGTCGTTAGCAT/ACCAGTTCATCAGCCTCAGC
LOX10/NM_001112510.1	CTTCAGCACCAAGCCAAGC/CCTCCTCCATTCACATCCAGA
PUBQ/NM_001154981.1	TAAGCCATCAGTCGTTGAAGC/CATGAAACCAGCTCAGTCACG
ATUB/NM_001111970.1	CCTTCAGCACCTTCTTCAGC/TTGTTAGCGGCATCCTCCTT

*Sequences were obtained from the National Center for Information Technology (NCBI, USA, at http://www.ncbi.nlm.nih.gov/)

recommendations. The assays were performed using three technical replicates, and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Different conditions of temperature and humidity can alter gene expression, due to this fact the experiments were conducted under controlled conditions, aiming to standardize abiotic conditions.

Olfactory behavior

The response of *T. pretiosum* to VOCs in maize uninfested and infested with *S. frugiperda* was assessed using a "Y" type olfactometer (diameter, 2.5 cm; main arm, 18 cm; smaller arms, 9 cm). The odor sources were placed inside glass containers (height, 70.0 cm, width, 25.0 cm, length, and 35.0 cm), to which the ends of the olfactometer were connected. The main arm of the olfactometer was connected to a vacuum pump. Airflow was adjusted to 300 mL/min using calibrated flow meters connected to each arm. Mated females 2–3 days old were individually positioned at the beginning of the central arm of the "Y" tube and observed for 5 min regarding choosing odor sources. It was considered a choice when the wasps reached the middle of an arm and remained in the arm for 10 s. Insects that did not choose an arm during the 5-min period were considered non-responsive and were excluded from the analysis. Each parasitoid was used only once to prevent odor learning. Every ten wasps released, the olfactometer was disassembled, and glassware was washed with mild soap, distilled water, and 90% alcohol (v/v), and rotated 180 °. The olfactometry tests were performed in the laboratory, under controlled conditions (25 ± 5 °C; 70 ± 15% relative humidity (RH); 14:10 (light (L):dark (D)). Each experiment was replicated three times, and at least 70 wasps were tested per treatment.

To evaluate the response of parasitoids to HIPV´s and potential interferences from equipment and external factors, the following bioassays were performed: (i) clear air vs. clear air, (ii) clear air vs. undamaged plant, and plant with clip cage vs. plant without clip cage. The following hydrids combinations were evaluated: (iii) DKB390 (isogenic line) vs. DKB390 VTPRO, (iv) DKB390 (isogenic line) vs. DKB390 VTPRO2, (v) DKB390 (isogenic line) vs. DKB390 VTPRO3, (vi) DKB390 (isogenic line) vs. Ag 3700 RR2, including UD (undamaged, control), plants subjected to DH (8:00 a.m. to 2:00 p.m.), plants subjected to NH (2:00 a.m. to 8:00 a.m.), and plants subjected to 24-h H (8:00 a.m. to 8:00 a.m. the following day). The assays were performed in the laboratory between 9:00 a.m. and 5:00 p.m.

Plant volatile collection and chemical analyses

Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC/MS) was used. The composition of VOCs emitted by plants subjected to DH, NH, or 24-h H, undamaged plants and negative control (empty glass vases) was evaluated (totaling 75 samples). For this purpose, after infestations, larvae were removed before the beginning accumulation of volatiles. The pots with the plants were wrapped in aluminum foil to reduce volatile emission from the roots and soil. Then, the plants were enclosed in a 2 L glass vase and completely sealed. After 2 h of accumulation of volatiles, a fiber (SPME assembly Divinylbenzene/Carboxen/Polydimethylsiloxane [DVB/CAR/PDMS], Sigma-Aldrich®) was inserted in the vial for the absorption/absorption of volatiles and remained in the vial for 60 min. After this period, the fiber was inserted into the injector of the GC/MS gas chromatograph (Thermo® Finnigan Trace). Relative quantification was performed according to the total peak area. VOCs were identified by comparing the mass spectra obtained from the NIST/EPA/NIH (2011) databases. Assays were performed under laboratory-controlled conditions.

Statistical analysis

LAC and gene expression data were subjected to analysis of variance (ANOVA), followed by Tukey's HSD at level of significance of 5%. Odor preference data were subjected to chi-square tests for categorical data (Crawley 2013). Data for individuals that made no choice within 5 min were excluded from the analyses. Volatile organic compounds (VOC) data

were transformed [log (x + 0.5)] for multivariate analysis of variance (MANOVA). Principal component analysis (PCA) was performed. All analyses were performed using R software version 3.0.2 (R Development Core Team 2014).

Results

Leaf area consumption (LAC)

The amount of plant tissue consumed by *S. frugiperda* larvae varied between hybrids ($F_{(4, 100)} = 8.958$, $p < 0.001$); Fig. 1a) and infestation time ($F_{(3, 100)} = 6.939$, $p < 0.001$; Fig. 1b). The hybrid Ag 3700 RR2 and DKB390 (isogenic) showed higher LAC compared to the maize varieties containing Cry proteins. Nonetheless, there were no significant differences in LAC between Bt plants (Fig. 1a). Larvae showed higher feeding activity during NH and 24-h H (Fig. 1b).

Analysis of differential gene expression

The analysis of hybrids with singular and multiple traits under infestation with *S. frugiperda* indicated that the expression of the TPS10 gene was increased in damaged plants ($F_{(14, 30)} = 751.9$, $p < 0.001$; Fig. 2a). The expression of this gene was significantly higher in plants subjected to DH, NH, and 24-h H than in UD, and there were significant differences in feeding times between hybrids. In Ag 3700 RR2, VTPRO, VTPRO2, and VTPRO3, NH significantly increased the expression of the TPS10 gene (Fig. 2a). In the isogenic form DKB390, TPS10 expression was similar between DH and NH but significantly higher than during 24-h H. The expression pattern of TPS10 did not vary significantly between VTPRO, VTPRO2, and VTPRO3. However, TPS10 expression was significantly different between UD and plants under DH in GM hybrids compared with the isogenic form DKB390.

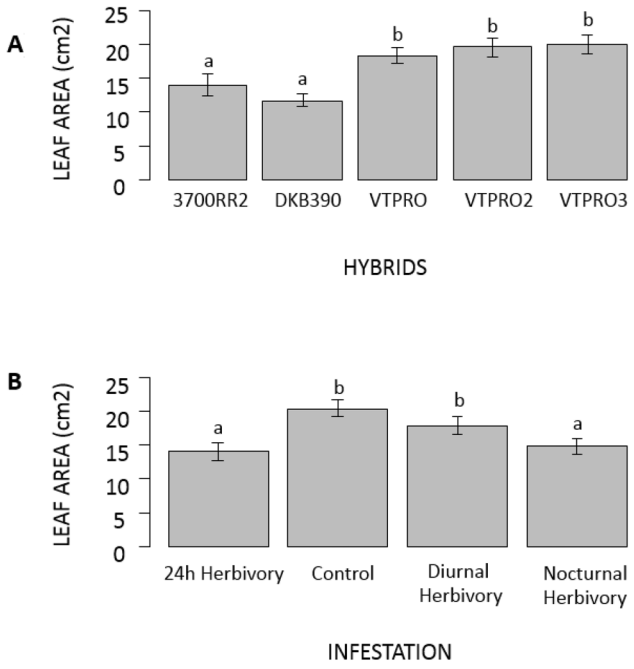

Fig. 1 Leaf area (cm^2) consumed by *Spodoptera frugiperda* in corn (*Zea mays* L.). **a** In single maize hybrids (Ag3700 RR2, DKB390 VTPRO), stacked (DKB390 VTPRO2 and DKB390 VTPRO3) and isogenic line DKB390. **b** Infestation (24-h H, from 8:00 a.m to 8:00 a.m; diurnal herbivory, from 8:00 a.m to 2:00 p.m; nocturnal herbivory, from 2:00 a.m to 8:00 a.m, and control (undamaged)). Means followed by the same letter do not differ by Tukey's HSD ($p < 0.05$). Error bars represent SE of the mean. For each treatment, three replications were considered. ($N = 3$)

The expression of the TPS23 gene was significantly different between treatments (Fig. 2b). ($F_{(14, 30)} = 738.7$, $p < 0.001$). In VTPRO, VTPRO2, VTPRO3, and the isogenic form, gene expression was higher under NH. However, in Ag 3700 RR2, there were no significant differences in TPS23 expression between feeding times. The expression pattern of this gene in Bt hybrids was similar to that in TPS10; however, the expression levels of the former were lower. Moreover, TPS23 was not detected in UD.

There were significant differences in the expression of the LOX gene between feeding times ($F_{(14, 30)} = 738.7$, $p < 0.001$; Fig. 2c) and hybrids. The expression of this gene was lower in UD, controls, and plants under DH. LOX expression was higher in plants under 24-h H than in UD and plants under DH. Furthermore, LOX expression was higher in DKB390, VTPRO, VTPRO2, and VTPRO3 during NH. However, in Ag 3700 RR2, the expression of this gene was not significantly different between NH and 24-h H. The expression pattern of LOX was similar between the Bt hybrids with single and multiple traits.

The expression pattern of the STC1 gene was different from that of the other genes. Although there were significant differences in expression between feeding times ($F_{(19, 40)} = 126.7$, $p < 0.001$; Fig. 2d), STC1 expression was lower in VTPRO and DKB390 in all feeding periods and higher in VTPRO2 and VTPRO3 under NH. In herbicide-tolerant Ag 3700 RR2, STC1 expression was higher in UD and lower in plants under NH, indicating that *S. frugiperda* feeding decreased the expression of this gene in this cultivar. The overall pattern of STC1 expression in hybrids with two Bt proteins (VTPRO2) or three Bt proteins (VTPRO3) was different from that in the isogenic form DKB390.

Olfactory behavior

The results of the equipment calibration tests (air vs. air, UD vs. air, and plant with clip cage vs. plant without clip cage) are presented in Fig. 3a–c, respectively, evidencing that there was no external interference in the behavior of the wasps

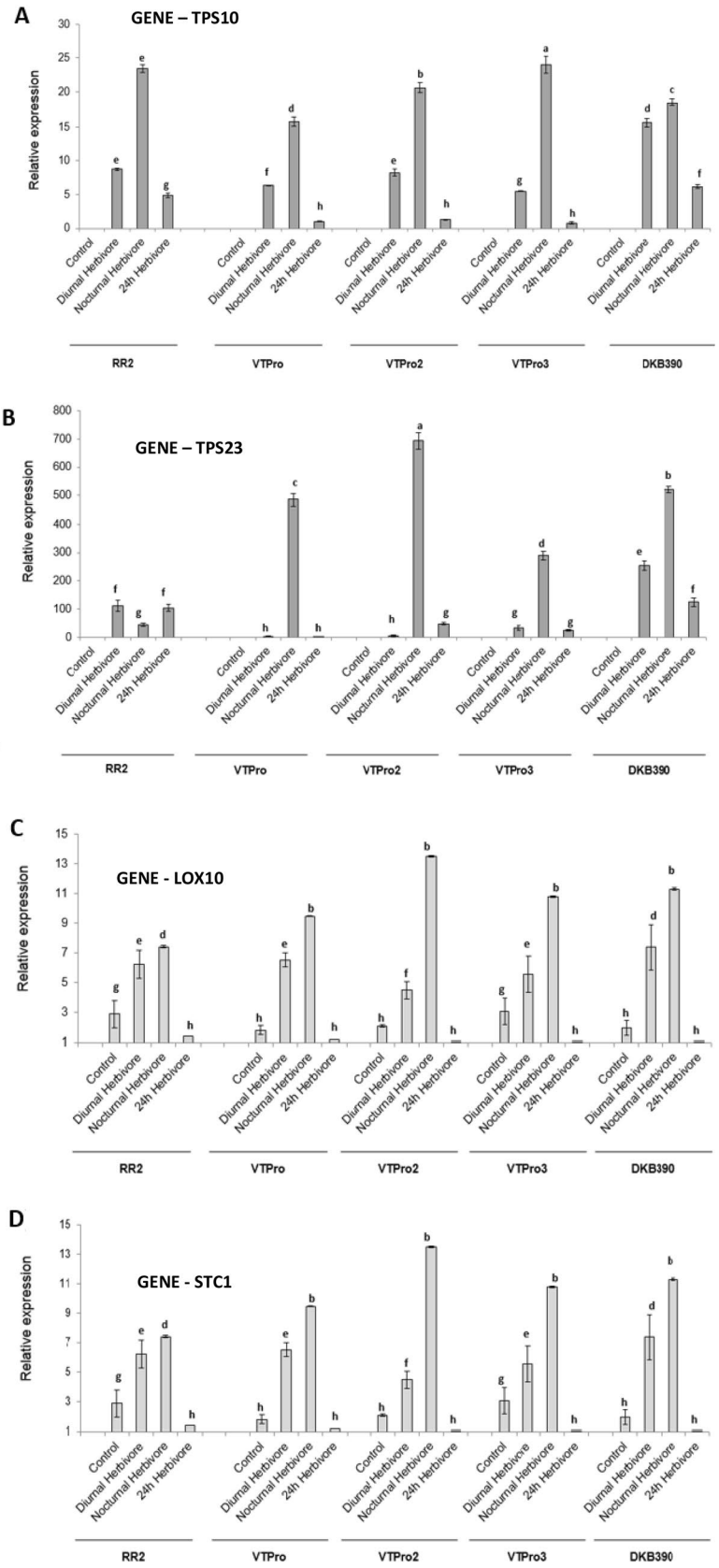

Fig. 2 Real-time quantitative PCR analysis of the relative abundance of **a** TPS10, **b** TPS23, **c** LOX10, and **d** STC1 gene transcripts in *Zea mays* L. undamaged (control) corn plants submitted to *Spodoptera frugiperda* herbivore in different hybrids and infestation schedules. Relative mRNA quantification was developed with PUBQ efficiency correction as a reference gene. Values are the average of three replicates normalized by expression of reference genes. Means followed by the same letter do not differ by Tukey's HSD ($p < 0.05$). Error bars represent SE of the mean. For each treatment, nine biological samples were considered. ($N = 9$)

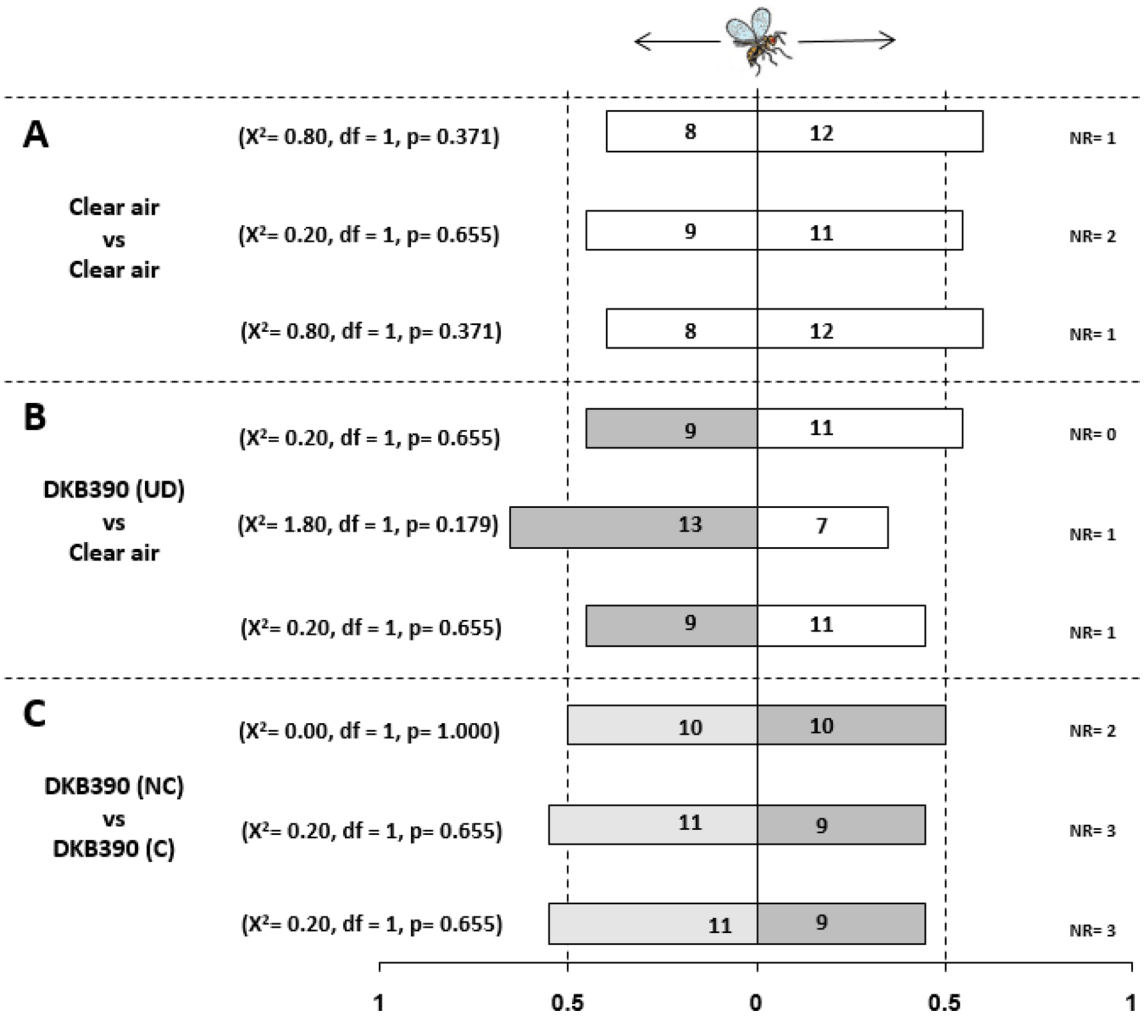

Fig. 3 Olfactory response of *Trichogramma pretiosum* females. Equipment calibration. The treatments were tested in pairs. The sources of odor consisted of the following: **a** clear air vs. clear air; **b** DKB390 (undamaged, UD) vs. clear air; **and c** DKB390 (no-clip cage, NC) vs. DKB390 (clip cage, C). NR represents non-responsive insects (no choice). Numbers in bars represent individual parasitoids that choose the indicated odor. *Significant at 5% according to chi-square; **significant at 1% according to chi-square. Up to 70 wasps were tested per treatment. For each treatment, three replications were performed ($N = 3$)

and that the clip cages did not induce the release of volatile compounds, through mechanical damage. The wasps were not attracted to VOCs induced by the controls of all hybrids (Figs. 4 and 5). Furthermore, the wasps were not attracted to volatiles induced in photophase under DH (diurnal herbivory, from 08:00 a.m. to 02:00 p.m., Fig. 6) and 24-h H (from 08:00 a.m. to 08:00 a.m., Fig. 7).

In contrast, adult females of *T. pretiosum* had a strong response to HIPV´s induced during NH (Fig. 5a–d). The volatiles induced in the isogenic form DKB390 were the most attractive.

Plant volatiles and chemical analyses

The five maize hybrids differed qualitatively and quantitatively in constitutive VOCs. Forty-four compounds were detected in hybrids with singular or multiple traits under DH, NH, and 24-h H, and UD (Table 2). VOCs similar to those found in previous studies were identified (Peñaflor et al. 2011; Leppik and Frérot 2014; Naranjo-Guevara et al. 2017; Coll et al. 2019). VOCs fall into five categories: aromatic hydrocarbons, terpenes, fatty acid derivatives, aldehydes, and salicylates.

VOC emission varied among hybrids and feeding conditions. For instance, the isogenic form DKB390 released four compounds under control conditions, eight during DH, six under 24-h H, and 18 during NH. In the single-trait cultivar DKB390 VTPRO, nine compounds were detected under control conditions, nine during DH, 14 under 24-h H, and 12 during NH. In DKB390 VTPRO2, seven compounds were released in UD, 10 during DH, 14 under 24-h H, and 13 during NH. In DKB390 VTPRO3, compounds were detected

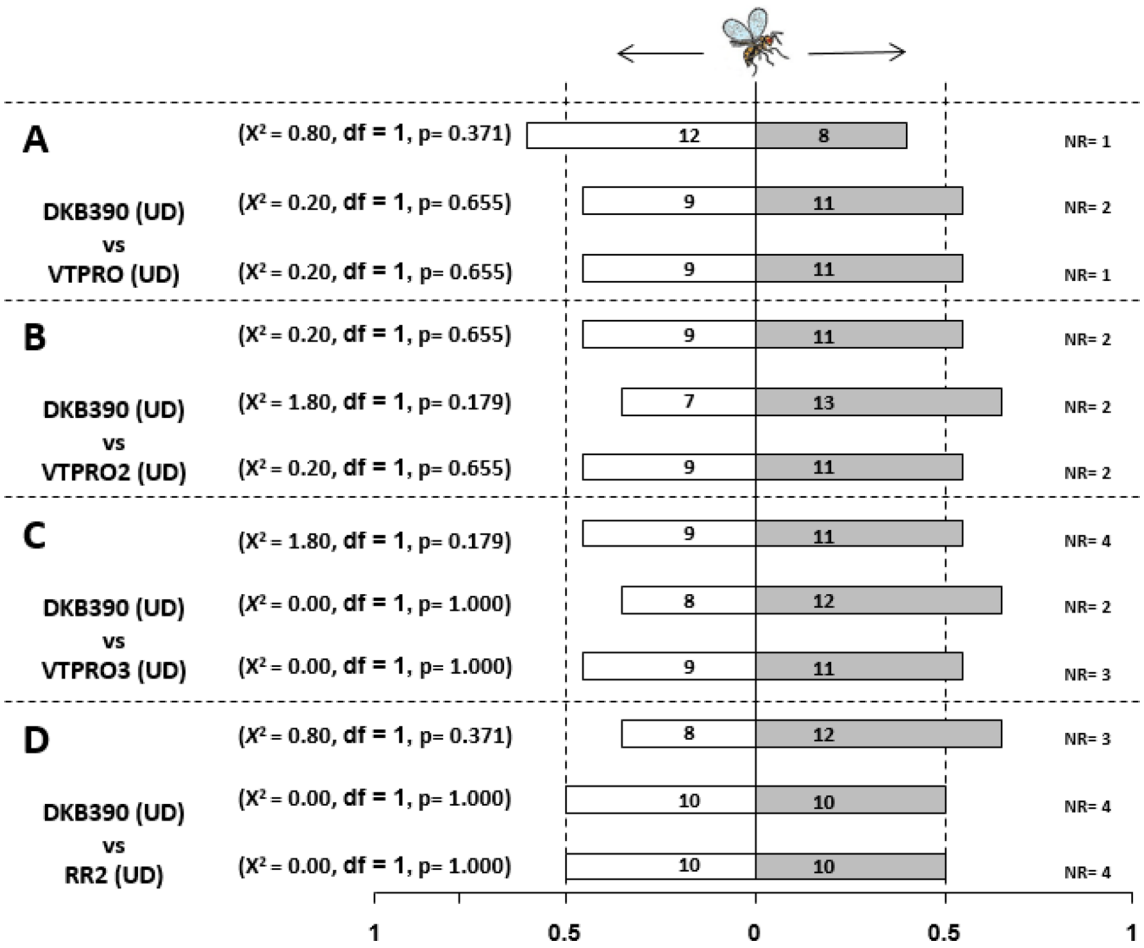

Fig. 4 Olfactory response of *Trichogramma pretiosum* females to constitutive volatiles. The treatments were tested in pairs. The sources of odor consisted of the following: **a** DKB390 DKB390 (undamaged, UD) vs. DKB390 VTPRO (undamaged, UD); **b** DKB390 (undamaged, UD) vs. DKB390 VTPRO2 (undamaged, UD); **c** DKB390 (undamaged, UD) vs. DKB390 VTPRO3 (undamaged, UD); **and d** DKB390 (undamaged, UD) vs. Ag 3700RR2 (undamaged, UD). NR represents non-responsive insects (no choice). Numbers in bars represent individual parasitoids that choose the indicated odor. * Significant at 5% according to chi-square; **Significant at 1% according to chi-square. Up to 70 wasps were tested per treatment. For each treatment, three replications were performed (N=3)

under control conditions, 10 during DH, seven under 24-h H, and 14 during NH. In Ag 3700 RR2, 12 compounds were identified in UD, 14 under DH, 10 during 24-h H, and 20 under NH.

In multiple comparisons between the five hybrids, seven VOCs were identified exclusively in the isogenic form DKB390 under NH, including linalol ($F_{(19, 40)} = 324.6$, $p < 0.001$) β-Gurjunene ($F_{(19, 40)} = 73.86$, $p < 0.001$), Calamene ($F_{(19, 40)} = 78.13$, $p < 0.001$), thujone ($F_{(19, 40)} = 45.26$, $p < 0.001$), (Z)-3-hexenyl acetate ($F_{(19, 40)} = 21.65$, $p < 0.001$), (E,E) α-farnesene ($F_{(19, 40)} = 11.2$, $p < 0$ .001), and methyl salicylate ($F_{(19, 40)} = 324.6$, $p < 0.001$).

Other VOCs were found exclusively in Ag 3700 RR2, including α-patchoulene ($F_{(19, 40)} = 63.43$, $p < 0.001$), 3-Methyl-decane ($F_{(19, 40)} = 43.88$, $p < 0.001$), phenethylacetate ($F_{(19, 40)} = 55.47$, $p < 0.001$), Indole ($F_{(19, 40)} = 7.833$, $p < 0.001$), (E)-3-hexenal ($F_{(19, 40)} = 97.6$, $p < 0.001$), (Z)-3-hexen-1-ol ($F_{(19, 40)} = 19.46$, $p < 0.001$), and geranyl acetate ($F_{(19, 40)} = 20.82$, $p < 0.001$). (Table 2). In addition, the levels of α-cadinene ($F_{(19, 40)} = 52.83$, $p<0.001$) and (Z)-3-hexenyl acetate differed significantly between treatments ($F_{(19, 40)} =21.65$, $p<0.001$) and were higher in Ag 3700 RR2 than in the isogenic line. The compounds (E)-2-hexenal, Thujone, δ-Amorphene, 3-Carene, camphene, (E, E)-4, 8, 12-Trimethyl-1, 3, 7, 11-tridecatetraene (TMTT), and (E)-nerolidol were found exclusively in Bt hybrids but were not sufficient to attract parasitoid wasps. Furthermore, compounds such as (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (E)-beta-Farnesene, α-Muurolene, Sabinene, 2-Isopropyl-5-methyl-9-methylene-bicyclo-1-decene[4.4.0], Decanal, and E-2-heptenal were not released under NH.

The PCA explained 58.8% of the total variation (Fig. 8). The first axis of the PCA corresponded to 20.1% of the total variation and was positively associated with terpenes. The

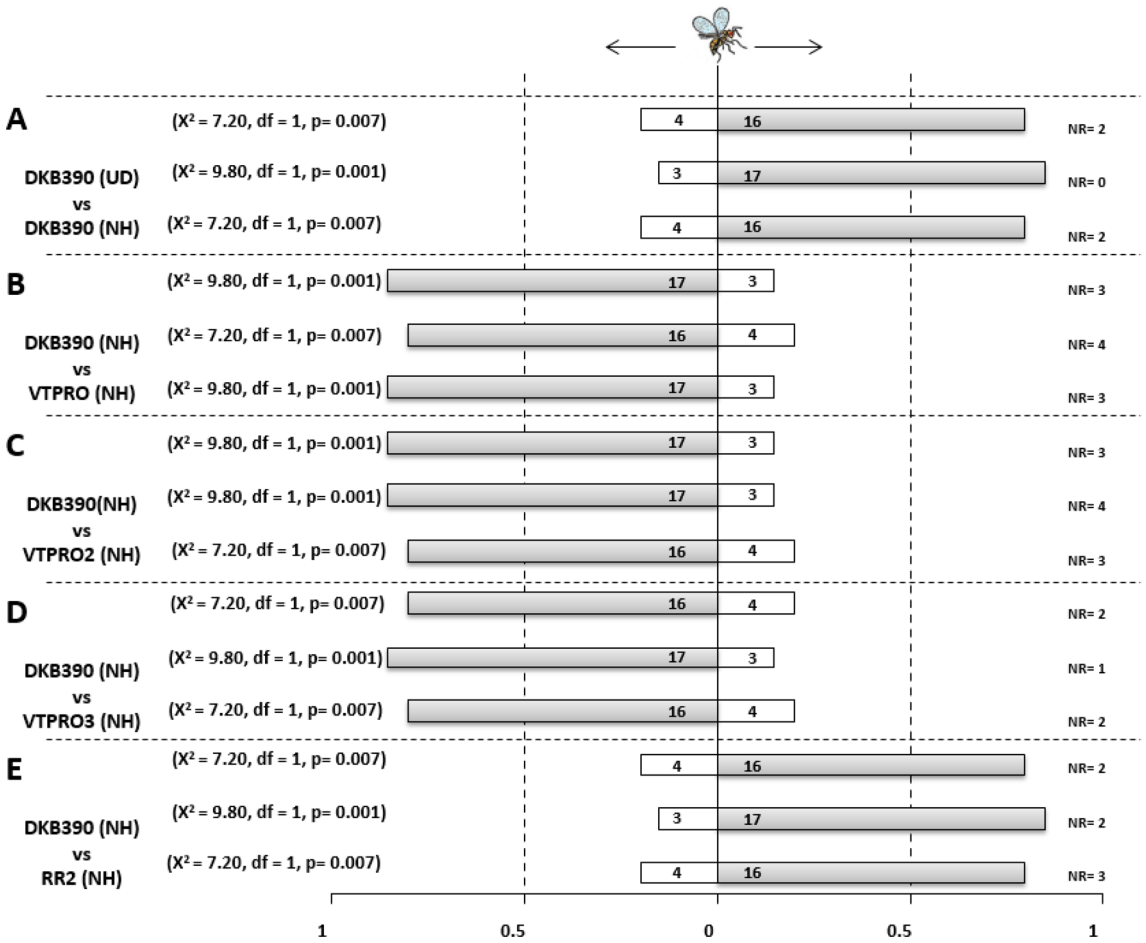

Fig. 5 Olfactory response of *Trichogramma pretiosum* females *to Spodoptera frugiperda*-induced volatiles in scotophase. The treatments were tested in pairs. The sources of odor consisted of the following: **a** DKB390 (undamaged, UD) vs. DKB390 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.); **b** DKB390 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.) vs. DKB390 VTPRO (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.); **c** DKB390 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.) vs. DKB390 VTPRO2 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.); **d** DKB390 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.) vs. DKB390 VTPRO3 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.); and DKB390 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.) vs. Ag 3700 RR2 (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.). NR represents non-responsive insects (no choice). Numbers in bars represent individual parasitoids that choose the indicated odor. Chi-square test ($p < 0.05$). Up to 70 wasps were tested per treatment. For each treatment, three replications were performed ($N = 3$)

second component explained 38.7% of the total variation and was positively correlated to aromatic hydrocarbons, fatty acid derivatives, aldehydes, and salicylates, and negatively correlated with terpenes.

Discussion

This study found significant differences in the attractiveness of the egg parasitoid *T. pretiosum* to HIPV´s in maize between diurnal and nocturnal infestations and considering transgenic and non-transgenic hybrids. *T. pretiosum* was more attracted to HIPV´s under nocturnal infestations in the isogenic form DKB390. In turn, the parasitoids were not attracted to HIPV´s from Bt plants, suggesting that Bt hybrids do not emit sufficient chemical cues to trigger host seeking by the parasitoid. However, HIPV´s released by Ag 3700 RR2 were more attractive than those released by DKB390 (isogenic line) under nocturnal infestations In contrast, the parasitoids were not attracted to VOC´s emitted under diurnal and 24-h herbivory.

The results of this study provide important information about the use of HIPV´s by egg parasitoids. It is known that eggs emit only small amounts of volatiles and are therefore only useful as short-range cues. Moreover, eggs generally are inconspicuous and deposited on hidden sites, making their location difficult (Vinson 1998; Turlings and Erb 2018). However, HIPV´s can be detected over long distance, making them important signals in the host finding behavior of parasitoids (Vet and Dicke 1992).

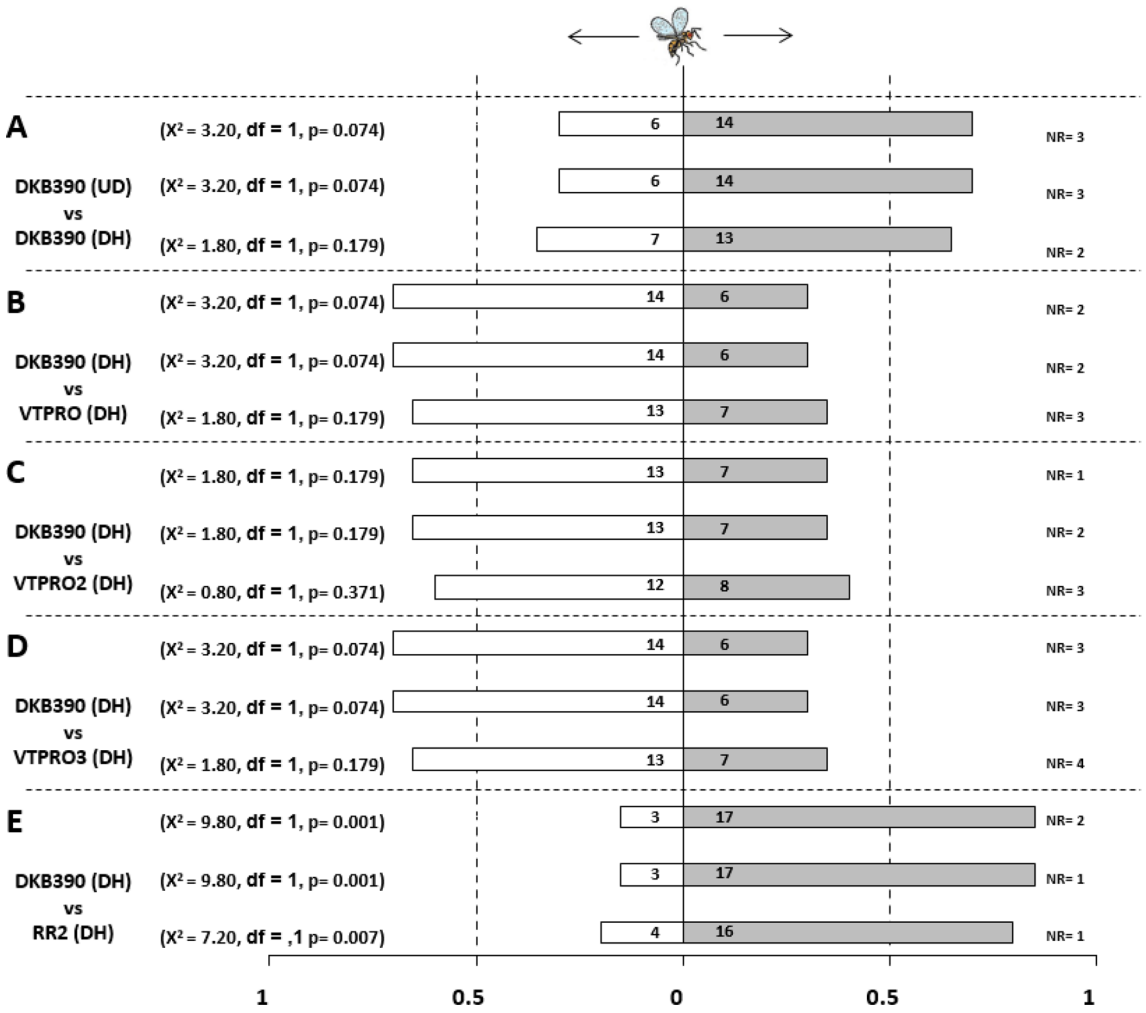

Fig. 6 Olfactory response of *T. pretiosum* females to *S. frugiperda*-induced volatiles in photophase. The treatments were tested in pairs. The sources of odor consisted of the following: **a** DKB390 (undamaged, UD) vs. DKB390 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.); **b** DKB390 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.) vs. DKB390 VTPRO (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.); **c** DKB390 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.) vs. DKB390 VTPRO2 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.); **d** DKB390 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.) vs. DKB390 VTPRO3 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.); and **e** DKB390 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.) vs. Ag 3700 RR2 (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.). NR represents non-responsive insects (no choice). Numbers in bars represent individual parasitoids that choose the indicated odor. Chi-square test ($p < 0.05$). Up to 70 wasps were tested per treatment. For each treatment, three replications were performed ($N = 3$)

While HIPV´s can provide specific information about the attacking herbivore, such as species and herbivore stage (Mumm et al. 2005), our work shows that they also may serve as indirect cues, possibly giving away the presence of more inconspicuous host stages like eggs (Colazza et al. 2004; Fatouros et al. 2005; Hilker and Meiners 2006; Hilker and Meiners 2010; Fatouros et al. 2012). This behavior may be advantageous to the egg parasitoids, the HIPV´s may be associated only with eggs under certain environmental situations, for example, when there is a high herbivore density, it is common to find egg masses and larvae occurring simultaneously on the same plants. Therefore, HIPV´s might play a role as long-range cues to egg parasitoid. Once wasps find the host community, host odors and subtle oviposition induced cues from neighboring plants may precisely indicate the presence of the host (Tamiru et al. 2011).

The results GC/MS showed significant differences in the volatiles released by undamaged plants DKB390 e 3700RR2. However, in the olfactometer assays wasps did not differentiate between volatiles. Therefore, there was no difference in choosing between these hybrids.

In other studies, olfactory responses of parasitoids to Bt and non-Bt plants were evaluated. Previous studies have reported that parasitic wasps did not differentiate between healthy undamaged Bt and non-Bt plants, showing that when

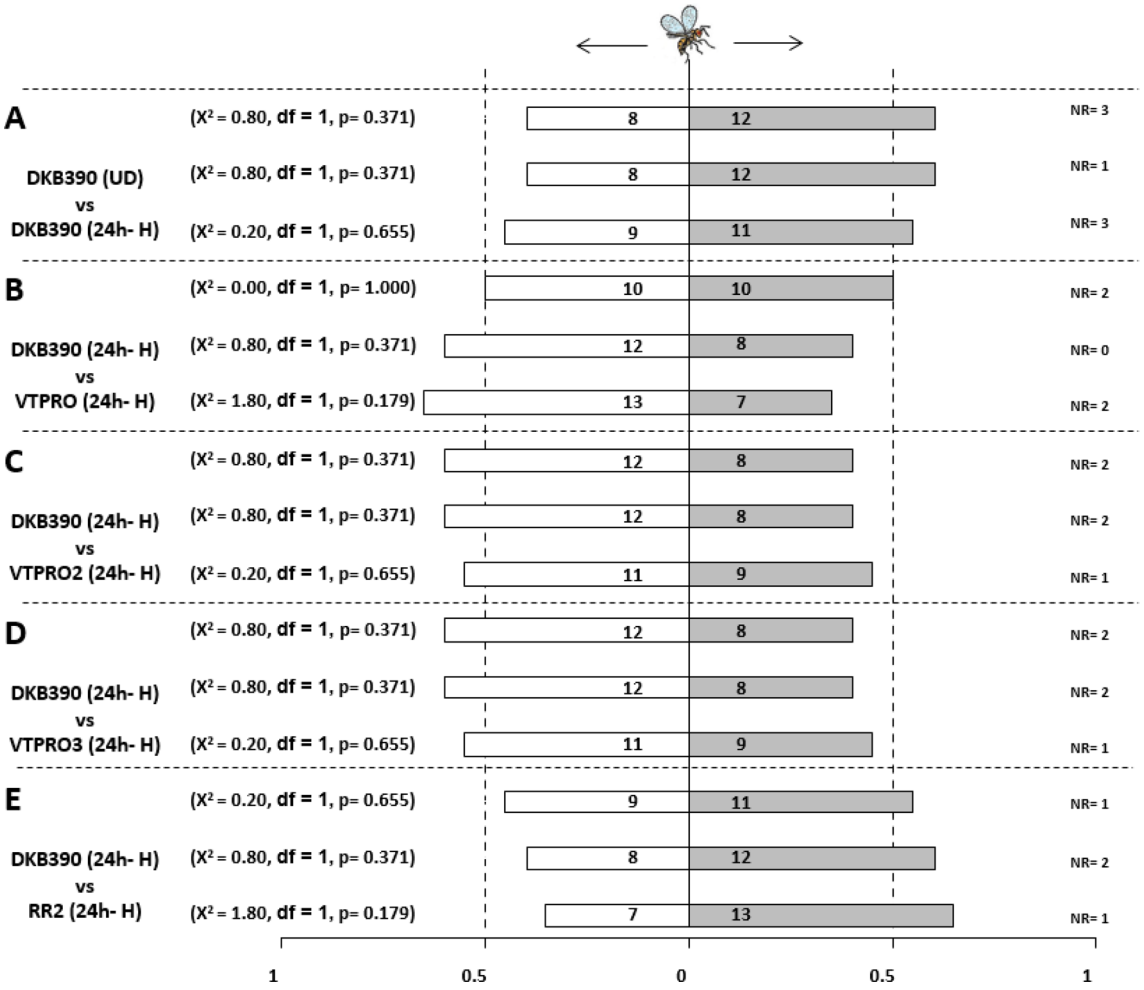

Fig. 7 Olfactory response of *T. pretiosum* females to *S. frugiperda*-induced volatiles in photophase. The treatments were tested in pairs. The sources of odor consisted of the following: **a** DKB390 (undamaged, UD) vs. DKB390 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.); **b** DKB390 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.) vs. DKB390 VTPRO (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.); **c** DKB390 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.) vs. DKB390 VTPRO2 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.); **d** DKB390 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.) vs. DKB390 VTPRO3 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.); and **e** DKB390 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.) vs. Ag 3700 RR2 (24 h of herbivory, 24-h H, from 08:00 to 08:00 a.m.). NR represents non-responsive insects (no choice). Numbers in bars represent individual parasitoids that choose the indicated odor. Chi-square test ($P < 0.05$). Up to 70 wasps were tested per treatment. For each treatment, three replications were performed ($N = 3$)

the plants are not under the attack of insects, the parasitoid does not distinguish between the GM and isogenic plant. Moraes et al. (2011) also reported that the egg parasitoid *T. pretiosum* showed an equal preference for undamaged Bt and non-Bt cotton plants, obtaining results similar to ours for tests with undamaged plants.

This present study, we evaluated Bt and non-Bt plants, and the variations within the genotype, if the insertion of one (VTPRO, RR2) or more proteins (VTPRO2, VTPRO3) affects the search behavior of *T. pretiosum*. Our results show that parasitoids are attracted to non-Bt plants when compared to Bt plants. Liu et al. (2015), demonstrated that the volatile-mediated interactions of rice plants with the parasitoid *Cotesia chilonis* were not disrupted by the genetic engineering of the plants. However, the greatest occurrence of the parasitoid in non-Bt rice is due to the damage and volatile induction by *Chilo suppressalis* larvae are greatly reduced in Bt fields. Similar results have been reported by Liu et al. 2015, that *C. chilonis* females were more attracted to rice plants damaged by 3rd-instar *C. suppressalis* larvae than to healthy rice plants. The authors explained that Bt rice plants are much less damaged than non-Bt plants, therefore, they release more volatile compounds that can be perceived by beneficial insects.

In experiments carried out with Bt and non-Bt rice, Wang et al. (2018) and Jiao et al. (2018) and found results

Table 2 Relative amounts of volatile emissions released by undamaged maize (UD) and maize damaged (DKB390 isogenic line, DKB390 VTPRO, DKB390 VTPRO2, DKB390 VTPRO3 and Ag 3700RR2) with *Spodoptera frugiperda* (diurnal herbivory, DH, from 08:00 a.m. to 02:00 p.m.), (nocturnal herbivory, NH, from 02:00 a.m. to 08:00 a.m.), and 24 hours of herbivory (from 08:00 a.m. to 08:00 a.m.). For each treatment, three replications were considered. ($N = 3$).

Compounds	DKB390				DKB390 VTPRO			
	UD	DH	NH	24-h	UD	DH	NH	24-h
(E)-2-hexenal	–	–	–	1.571 ± 1.62	1.376 ± 1.445	0.505 ±0.893	–	0.648 ± 0.968
(E)-3-hexenal	–	–	–	–	–	–	–	–
(Z)-3-hexen-1-ol	–	–	–	–	–	0.666 ± 0.999	–	–
Thujone	–	–	1.018 ± 0.915	–	–	–	–	-
(Z)-3-hexenyl acetate	–	–	0.601 ± 0.012	–	–	–	–	–
β-Myrcene	–	–	–	–	–	–	1.120 ± 0.009	–
trans-β-Caryophyllene	–	1.457 ± 0.698	–	–	0.3360 ± 0.998	1.176 ± 1.045	–	0.566 ± 0.008
(+)-Cyclosativene	–	1.140 ± 1.002	–	1.290 ± 0.008	1.233 ± 1.003	1.261 ± 1.007	1.579 ± 0.003	1.028 ± 1.003
Ylangene	–	–	0.064 ± 0.001	0.764 ± 0.002	0.601 ± 0.012	–	0.856 ± 0.004	–
α-guaiene	1.218 ± 0.698	1.254 ± 0.035	0.412 ± 0.010	–	–	–	0.455 ± 0.088	1.043 ± 1.004
β-curcumene	–	–	0.717 ± 0.992	–	–	–	–	–
α-patchoulene	–	–	0.643 ± 0.007	–	–	–	0.787 ± 0.006	0.890 ± 0.017
α-Muurolene	1.359 ± 1.091	–	–	1.187 ± 1.003	–	1.301 ± 1.005	0.747 ± 0.002	0.980 ± 0.100
α-cadinene	–	–	0.055 ± 0.004	–	–	–	–	0.871 ± 0.004
α-Copaene	–	0.559 ± 0.004	0.521 ± 0.003	–	–	0.280 ± 0.002	–	0.329 ± 0.004
β-cis-Ocimene	–	–	–	0.629 ± 0.832	–	–	0.593 ± 0.201	–
Linalool	–	–	1.265 ± 0.472	–	–	–	0.454 ± 0.143	–
(E)-α-bergamotene	–	0.962 ± 0.167	–	–	0.475 ± 0.001	–	–	0.580 ± 0.776
(TMTT)	–	–	–	–	1.212 ± 0.996	–	–	–
(DMNT)	–	–	–	–	–	–	–	–
(E)-beta-Farnesene	–	–	–	–	0.725 ± 0.385	–	–	–
geranyl acetate	–	–	–	–	–	–	–	0.580 ± 0.083
α-pinene	–	–	0.387 ± 0.112	–	–	1.308 ± 0.005	0.728 ± 0.144	0.691 ± 0.065
δ-Amorphene	–	–	–	–	–	–	0.522 ± 0.088	–
3-Carene	–	0.419 ± 0.123	–	–	–	0.622 ± 0.035	0.326 ± 0.100	–
Calamene	–	–	0.499 ± 0.122	–	–	–	–	–
β-Gurjunene	–	–	0.454 ± 0.083	–	–	–	–	–
Sabinene	1.402 ± 1.000	0.828 ± 0.088	–	–	–	–	–	–
(E)-nerolidol	–	–	–	–	–	–	–	–
1,2,4-trimethylbenzene	–	–	–	–	–	–	–	–
3-Methyl-decane	–	–	–	–	–	–	–	–
Camphene	–	–	0.360 ± 0.015	–	–	–	–	1.099 ± 0.083
Decanal	–	–	–	–	1.264 ± 1.063	–	–	–
Anisaldehyde	–	–	0.482 ± 0.067	–	–	–	–	–
E-2-heptenal	–	–	–	–	–	–	–	–
2,4-hexadienal	–	–	–	–	–	–	0.538 ± 0.267	–
Phenethyl acetate	1.526 ± 1.058	–	–	–	0.674 ± 0.145	–	–	-
(E,E)-α-farnesene	–	–	0.450 ± 0.020	–	–	–	—	–

Table 2 (continued)

Compounds	DKB390				DKB390 VTPRO			
	UD	DH	NH	24-h	UD	DH	NH	24-h
Indole	–	–	0.365 ± 0.004	–	–	–	–	–
Methyl salicylate	–	–	1.471 ± 1.003	–	–	–	–	–
UNKNOWN sesquiterpene	–	–	–	0.119 ± 0.034	–	–	–	–
UNKNOWN1	–	1.035 ± 0.099	–	0.673 ± 0.012	–	0.459 ± 0.088		0.162 ± 0.045
UNKNOWN2	–	–	0.837 ± 0.145	0.584 ± 0.034	–	–	–	0.584 ± 0.069
Compounds	DKB390 VTPRO2				DKB390 VTPRO3			
	UD	HD	HN	24 h	UD	HD	HN	24-h
(E)-2-hexenal	1.476 ± 1.537	1.433 ± 1.5	–	–	1.092 ± 1.227	–	1.541 ± 1.594	–
(E)-3-hexenal	–	–	–	–	–	–	–	–
(Z)-3-hexen-1-ol	0.741 ± 0.005	–		0.295 ± 0.005	–	–	–	–
Thujone	–	–	–	–	–	–	–	–
(Z)-3-hexenyl acetate	–	–	–	–	–		–	–
β-Myrcene	–	–	1.003 ± 1.234	–	–	–	–	–
trans-β-Caryophyllene	0.436 ± 0.004	0.783 ± 0.034	–	0.725 ± 0.011	0.723 ± 0.007	1.422 ± 0.009	0.599 ± 0.005	–
(+)-Cyclosativene	0.673 ± 0.004	–	–	0.183 ± 0.005	1.213 ± 1.004	1.365 ± 0.009	1.095 ± 0.078	1.295 ± 0.123
Ylangene	1.321 ± 0.698	–	–	0.222 ± 0.007	0.671 ± 0.045	–	–	–
α-guaiene	–	0.781 ± 0.007	0.462 ± 0.006	1.093 ± 1.392	0.501 ± 0.032	–	0.373 ± 0.008	1.219 ± 0.993
β-curcumene	–	–	–	–	–	–	–	–
α-patchoulene	–	–	0.919 ± 0.004	0.133 ± 0.834	–	0.965 ± 0.009	–	–
α-Muurolene	–	–	–	–	–	–	0.606 ± 0.007	
α-cadinene	–	–	–	1.249 ± 0.698	–	–	–	0.207 ± 0.022
α-Copaene	–	1.050 ± 1.003	–	–	–	0.664 ± 0.122	–	–
β-cis-Ocimene	–	1.188 ± 0.999	0.654 ± 0.023	–	–	–	0.429 ± 0.012	–
Linalool	–	–	0.207 ± 0.006	–	–	–	0.172 ± 0.807	–
(E)-α-bergamotene	1.081 ± 1.004	–	0.774 ± 0.011	–	0.814 ± 0.303	–	0.743 ± 0.170	1.003 ± 0.654
(TMTT)	–	–	1.137 ± 0.765	–	–	–	–	–
(DMNT)	–	–	–	–	–	–	–	–
(E)-beta-Farnesene	–	–	–	–	–	–	–	–
geranyl acetate	–	–	–	–	1.225 ± 1.004	1.073 ± 0.999	–	–
α-pinene	1.321 ± 0.045	1.383 ± 1.056		0.595 ± 0.178	0.967 ± 0.06	5 1.136 ± 1.003	- -	
δ-Amorphene	–	–	–	–	–	0.434 ± 0.056	–	–
3-Carene	–	–	–	–	–	–	–	0.352 ± 0.006
Calamene	–	–	–	–	–	–	–	–
β-Gurjunene	–	–	–	0.466 ± 0.034	–	–	–	–
Sabinene	–	–	–	1.178 ± 1.102	–	–	–	–
(E)-nerolidol	–	–	–	–	–	–	0.488 ± 0.083	0.172 ± 0.045

Table 2 (continued)

Compounds	DKB390 VTPRO2				DKB390 VTPRO3			
	UD	HD	HN	24 h	UD	HD	HN	24-h
1,2,4-trimethylbenzene	–	–	–	0.161 ± 0.023	–	–	0.301 ± 0.034	–
3-Methyl-decane	–	–	–	–	–	–	–	–
Camphene	–	0.986 ± 0.098	0.060 ± 0.004	0.060 ± 0.004	–	–	–	–
Decanal	–	–	–	–	-	–	–	–
Anisaldehyde	–	1.103 ± 1.005	0.282 ± 0.056	0.485 ± 0.034	–	–	0.649 ± 0.145	–
E-2-heptenal	–	–	–	–	–	–	–	–
2,4-hexadienal	–	–	–			0.530 ± 0.045	1.231 ± 1.011	–
Phenethyl acetate	1.252 ± 1.098	–	–	–	–	–	–	–
(E,E)-α-farnesene	–	–	–	–	–	–	–	–
Indole	–	–	–	0.526 ± 0.012	–	–	–	–
Methyl salicylate		–	–	–	–	–	–	–
UNKNOWN sesquit-erpene	–	–	–	–	1.134 ± 1.004	–	–	–
UNKNOWN1	–	0.029 ± 0.003	0.489 ± 0.178	0.029 ± 0.003	–	0.435 ± 0.034	1.008 ± 0.999	–
UNKNOWN2	–	0.881 ± 0.088	0.056 ± 0.003	–	–	0.807 ± 0.069	0.881 ± 0.086	0.977 ± 0.178

Compounds	Ag3700RR2			
	UD	DH	NH	24-h
(E)-2-hexenal	1.160 ± 1.279		0.129 ±1.06	–
(E)-3-hexenal	–	–	0.147 ± 0.776	–
(Z)-3-hexen-1-ol	–	0.874 ± 0.004	1.369 ± 1.003	–
Thujone	–	–	–	–
(Z)-3-hexenyl acetate	–	–	–	–
β-Myrcene	–	–	–	–
trans-β-Caryophyllene	0.722 ± 0.006	–	0.085 ± 0.004	–
(+)-Cyclosativene	1.075 ± 1.003	0.588 ± 0.003	0.383 ± 0.001	–
Ylangene	0.950 ± 0.005	–	–	–
α-guaiene	–	–	1.309 ± 0.721	1.075 ± 1.017
β-curcumene	–	–	–	–
α-patchoulene	–	0.965 ± 0.009	1.038 ± 0.088	–
α-Muurolene	–	0.384 ± 0.069	–	1.023 ± 0.996
α-cadinene	0.344 ± 0.007	–	1.182 ± 0.095	–
α-Copaene	–	0.156 ± 0.034	0.099 ± 0.001	0.451 ± 0.012
β-cis-Ocimene	–	–	0.072 ± 0.001	–
Linalool	–	–	1.355 ± 0.886	–
(E)-α-bergamotene	0.915 ± 0.142	–	–	–

Table 2 (continued)

Compounds	Ag3700RR2			
	UD	DH	NH	24-h
(TMTT)	–	–	–	–
(DMNT)	–	–	–	1.323 ± 1.043
(E)-beta-Farnesene	0.991 ± 0.112	–	–	–
geranyl acetate	0.523 ± 0.145	0.124 ± 0.034	0.444 ± 0.0340	0.366 ± 0.004
α-pinene	1.062 ± 1.002	1.235 ± 1.006	0.140 ± 0.045	0.206 ± 0.104
δ-Amorphene	–	0.581 ± 0.034	–	–
3-Carene	–	0.499 ± 0.234	1.067 ± 1.002	0.390 ± 0.004
Calamene	–	–	–	–
β-Gurjunene	–	–	–	–
Sabinene	0.156 ± 0.034	–	–	–
(E)-nerolidol	–	–	–	–
1,2,4-trimethylbenzene	–	–	–	–
3-Methyl-decane	–	–	0.029 ± 0.003	–
Camphene	–	0.586 ± 0.111	0.221 ± 0.004	–
Decanal	0.523 ± 0.145	–	–	–
Anisaldehyde	–	0.350 ± 0.056	–	0.699 ± 0.145
E-2-heptenal	–	0.253 ± 0.005	–	–
2,4-hexadienal	–	1.124 ± 1.023	–	–
Phenethyl acetate	1.070 ± 1.001	–	0.297 ± 0.008	–
(E,E)-α-farnesene	–	–	–	–
Indole	–	–	0.951 ± 0.023	–
Methyl salicylate	–	–	–	–
UNKNOWN sesquiterpene	–	–	0.204 ± 0.002	0.332 ± 0.034
UNKNOWN1	–	0.309 ± 0.005	–	0.638 ± 0.123
UNKNOWN2	–	–	0.557 ± 0.189	–

similar to this study, since the amount of volatiles detected in non-Bt plants was greater than Bt plants, due to the significantly greater caterpillar damage on non-Bt plants. In addition, GC/MS analyzes showed that caterpillar damage induced the release of rice plant volatiles known to be attractive to planthoppers and larval damage induced the release of volatiles that repelled mated *C. suppressalis* females.

After herbivore damage, biochemical reactions produce a systemic response in the plant at various levels, including changes in gene expression and synthesis of chemical compounds (Turlings et al. 2000; Turlings and Matthias 2018; Ton et al. 2007). A possible explanation for the higher attractiveness of parasitoids to HIPV´s emitted under nocturnal herbivory would be the higher expression of critical genes involved in the activation of plant defenses. In this respect, the relative expression of the genes TPS10, TPS23, STC1, and LOX10 was higher in plants under nocturnal herbivory (Figs. 2, 3, 4, and 5).

At the onset of damage, with the breakdown of the cell wall caused by feeding and the action of elicitors, LOX enzymes have a prominent role, stimulating the biochemical pathway that culminates in the activation of enzymes involved in the production of several VOCs (Nemchenko et al. 2006). This process is followed by the production of high levels of jasmonic acid (JA), which is a signaling molecule released by plants in response to only by chewer herbivores and mechanical damage (Schmelz et al. 2007; Wasternack and Hause 2013). JA triggered by LOX may be involved in the activation of enzymes that lead to the differential expression of other genes associated with plant defense. Studies show that LOX participates in protein activation and release of compounds indirectly involved in plant defenses by attracting herbivore predators (Paré and Tumlinson 1997a, b; Turlings and Matthias 2018). The high levels of JA promote an increase in the expression of early and late genes (Vandenborre et al. 2009), activating the production of several VOCs. The activation of the JA pathway by herbivore attack has been reported in *S. frugiperda* (Schmelz et al. 2007). JA-treated plants are more attractive to predators and parasitoids under laboratory and field conditions (Dicke et al. 1999).

The relative expression of the LOX10 gene was significantly higher than of the other genes. This result may be related to the fact that LOXs are not only involved in multiple functions in the plant cell but also directly related to the production of GLVs, which are released in the first hours of herbivore attack. In contrast, the expression of genes directly involved in the synthesis of volatile terpenes attractive to herbivores is lower (Turlings et al. 1998).

The results of gene expression were corroborated by the profile of the emission of (E)-β-farnesene, (E)-α-bergamotene, and other sesquiterpenes, whose production is regulated by the expression of the TPS10 and TPS23 genes (Schnee et al. 2002). This result suggests that the higher expression of these genes in herbivore-damaged plants compared to undamaged plants is directly linked to the activation of genes involved in the production of signaling compounds. In addition to these genes, studies demonstrate that herbivore attack increased the production of naphthalene and volicitin by increasing the expression of the STC gene (Shen et al. 2000; Lawrence and Novak 2004; Mérey et al. 2011).

The higher attractiveness to volatiles plants induced herbivore (HIPV´s) released by the isogenic cultivar relative to Bt hybrids can be explained by the higher emission of VOCs during scotophase and the exclusive production of some compounds, including thujone, (Z)-3-hexenyl acetate, Calamene, β-Gurjunene, Methyl salicylate, α-cadinene, α-Copaene, and Propylbenzene (Table 1). However, the results of olfactometry assays indicated that Ag 3700 RR2 released a mixture of HIPV´s that were more attractive to parasitoid wasps. (E)-2-hexenal, (E)-3-hexenal, (Z)-3-hexen-1-ol, trans-β-Caryophyllene, α-guaiene, β-cis-Ocimene, geranyl acetate, 3-Carene, 3-Methyl-decane, and Nonanal were found exclusively in Ag 3700 RR2.

Earlier studies show that nocturnal herbivory and diurnal herbivory result in differences in the production and emission of HIPV´s (De Moraes et al. 2001; Naranjo-Guevara et al. 2017). For instance, the pattern of increase in JA levels in maize plants under nocturnal herbivory is similar to that of plants under diurnal herbivory. However, the emission of sesquiterpenes and aromatic compounds is different under these conditions. Sesquiterpene release is lower at night and higher during the day (Schmelz et al. 2003). In contrast, other VOCs are emitted exclusively during the day (De Moraes et al. 2001; Naranjo-Guevara et al. 2017).

Signoretti et al. (2012) found similar results in *Campoletis flavicincta* regarding the response to HIPV´s produced by maize plants treated with a *S. frugiperda* regurgitate at night, indicating that the parasitoid was more attracted to HIPV´s released at night compared to those released during the day. Although there were no qualitative differences in the compounds between feeding times, their quantities was higher at night. In contrast, Arimura et al. (2008) found that the composition of HIPV´s in lima bean plants was significantly different under diurnal herbivory and nocturnal herbivory, and JA levels were higher under nocturnal herbivory.

The results of behavioral assays showed that the parasitoids preferred HIPV´s produced by plants under nocturnal herbivory. The LAC assay was performed to evaluate whether the feeding behavior of *S. frugiperda* larvae differed between photophase and scotophase. The results showed that LAC increased at night, which may also explain the increased emission of HIPV´s under nocturnal herbivory. Sparks (1979) have shown that *S. frugiperda* larvae feed more actively at night under field conditions because the

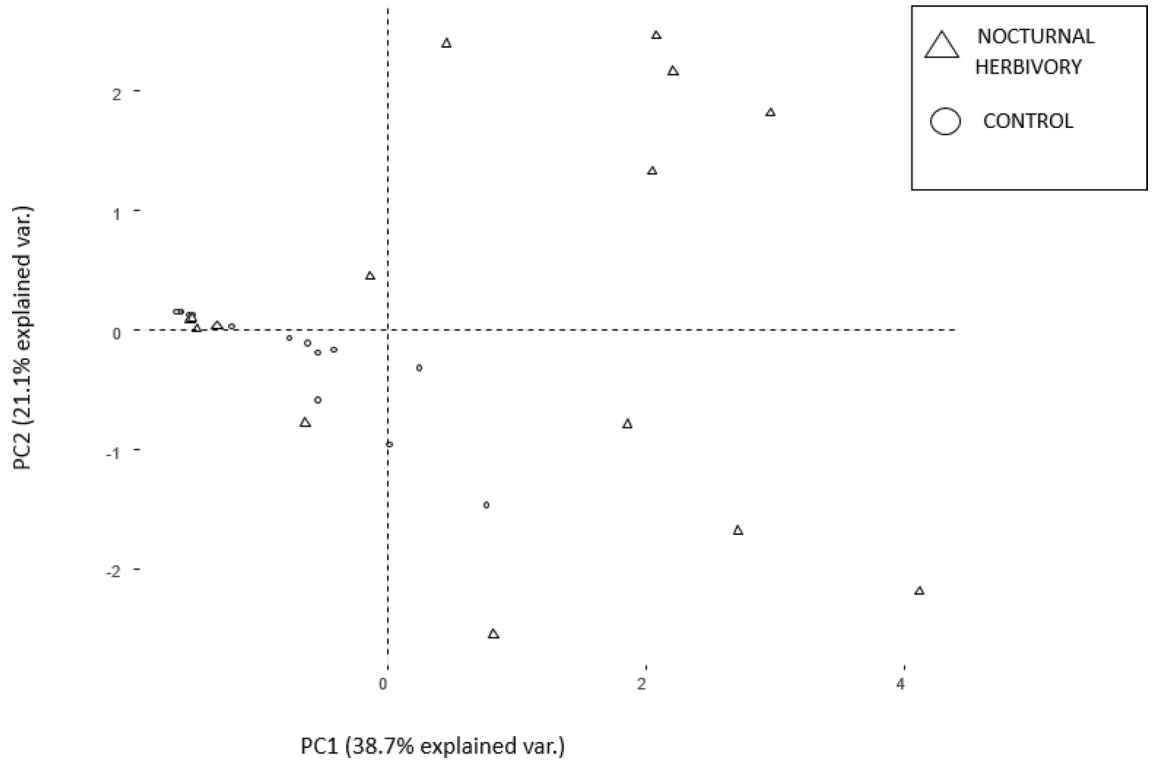

Fig. 8 Score plot for principal component analysis (PCA) for the composition of volatiles emitted by maize plants, control, (DKB390 isogenic, DKB390 VTPRO, DKB390 VTPRO2, DKB390VTPRO3 and Ag3700RR2), and nocturnal herbivory, NH, (DKB390 isogenic, DKB390 VTPRO, DKB390 VTPRO2, DKB390VTPRO3 and Ag3700RR2). The first two axes account for 38.7 and 20.1% of the total variation

temperature is lower, favoring insect activity. Therefore, in the evolutionary context, parasitoid wasps may have adapted to associate the presence of volatiles produced at night with the presence of the host (Signoretti et al. 2012).

The results of 24-h herbivory suggested that wasps were not attracted to the host because the amount of HIPV´s was lower in this period. In maize, volatile production may be very low or even absent over time. On average, the overall production of VOCs in maize begins to decrease after 12 h of pest attack (Turlings et al. 1998).

Conclusions

We found that the expression of the TPS10, TPS23, LOX10, and STC1 genes were increased in nocturnal infestations. The compounds thujone, (Z)-3-hexenyl acetate, calamene, β-Gurjunene, Methyl salicylate, α-cadinene, α-Copaene, and Propylbenzene, in addition to linalol, which were emitted only under nocturnal infestations and in larger amounts in the isogenic form DKB390, might be crucial for attracting *T. pretiosum*. Furthermore, the emission of HIPV´s in undamaged Bt plants was different from that in the isogenic form. Bt plants under nocturnal herbivory released fewer HIPV´s than the isogenic form. Furthermore, the number of constitutive compounds and compounds induced by *S. frugiperda* larval feeding at night was higher in non-Bt Ag 3700 RR2 than in non-Bt DKB390. We believe that the attractiveness of the parasitoid was higher in non-Bt plants, because less damage and HIPV´s emission took place in Bt plants. This study increases knowledge about biological pest control, tritrophic interactions, and current plant technologies.

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Authors contribution PTN planned, designed, and executed experimental work. MSR contributed with executed experimental. JOFM contributed with GC-MS analysis. Beatriz A. Barros contributed with RT-qPCR analysis. MAMF conducted data analyses. PTN wrote the manuscript. FHV, MAMF, CSFS, and RGVP reviewed the manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no competing interests.

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