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The parasitoid *Dolichogenidea gelechiidivoris* eavesdrops on semiochemicals from its host *Tuta absoluta* and tomato

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Abstract

Semiochemicals such as herbivore-induced plant volatiles (HIPVs) and host chemicals serve as communication signals for parasitoids searching for oviposition sites. The braconid koinobiont endoparasitoid *Dolichogenidea gelechiidivoris* (Hymenoptera: Braconidae) efficiently parasitises larvae of *Tuta absoluta* (Lepidoptera: Gelechiidae), a major pest of tomato (*Solanum lycopersicum*). However, the attractive compounds used by the parasitoid to locate *T. absoluta* on host plants are not known. We therefore performed behavioural assays and chemical analyses to investigate the chemical basis of interactions between the parasitoid, the tomato plant and *T. absoluta*. Y-tube olfactometer bioassays revealed that *D. gelechiidivoris* was attracted to *T. absoluta* larvae-infested tomato plant volatiles and preferred volatiles of plants with a high infestation level than those with a low infestation level. The parasitoid was also attracted to volatiles of larval frass and to the sex pheromone of *T. absoluta*. Coupled gas chromatography–mass spectrometric analyses were performed on plant and frass volatiles. We found both qualitative and quantitative differences in volatile emission between healthy and *T. absoluta* larvae-infested tomato plants, where volatile emission rate increased with increasing infestation level. The most characteristic volatile compounds which distinguished *T. absoluta* larvae-infested plants from healthy plants were α-pinene, sabinene, β-myrcene, 2-carene, α-phellandrene, 3-carene, α-terpinene, β-phellandrene, (Z)-β-ocimene, (E)-β-ocimene, allo-ocimene, (E)-β-caryophyllene and methyl salicylate. With the exception of caryophyllene oxide, all larval frass volatile compounds were also found in tomato plant headspace volatiles. Olfactometer bioassays using synthetic compounds revealed that *D. gelechiidivoris* was attracted to α-pinene, β-myrcene, α-phellandrene, α-terpinene, β-ocimene, methyl salicylate and (E)-β-caryophyllene, and the 7-component blend of these attractants elicited the greatest attraction in the parasitoid. These findings open new avenues for exploiting these attractants as kairomone-based lures to recruit and retain the parasitoid in tomato fields for the biological control of *T. absoluta*.

Keywords Biological control · HIPVs · Kairomone · Frass · Olfactometer · Leafminer

Key message

- We investigated semiochemicals mediating interactions between the braconid parasitoid *Dolichogenidea gelechiidivoris, Tuta absoluta*, and tomato plants.
- *Dolichogenidea gelechiidivoris* was attracted to volatiles of *T. absoluta larvae*-infested tomato plants, larval frass, and sex pheromone of *T. absoluta*.
- The blend of α-pinene, β-myrcene, α-phellandrene, α-terpinene, β-ocimene, methyl salicylate and (E)-β-caryophyllene was highly attractive to *D. gelechiidivoris*.
- The attractive volatile blend and the sex pheromone could be deployed as kairomone-based lures to recruit

and retain the parasitoid for biological control of *T. absoluta*.

Introduction

Kairomones are semiochemical molecules that are emitted by an organism to mediate interspecific interaction beneficial to the receiver organism (Dicke and Sabelis 1988; Kost 2008). Kairomones exploited by natural enemies are either plant volatiles or herbivore-associated chemicals such as pheromones (Afsheen et al. 2008; Turlings and Erb 2018; Ayelo et al. 2021). Herbivore-induced plant volatiles (HIPVs) are released by plants in response to herbivore feeding, and serve as long-range kairomones for parasitoids searching for hosts (Kessler and Baldwin 2001; Kaplan 2012; Turlings and Erb 2018; Ayelo et al. 2021). The HIPVs are a blend of compounds belonging to different chemical groups, while parasitoid attracting-compounds mainly belong to green leaf volatile and terpenoid groups (Mumm and Dicke 2010; Turlings and Erb 2018). The herbivore feeding behaviour, i.e. chewing or sap sucking, determines which plant defence pathway (jasmonic acid or salicylic acid) will be activated, and this influences the emission of HIPVs (Danner et al. 2018). Other biotic factors including the herbivore-infesting species, the level of infestation, the infesting instar, and the host plant species and genotype shape the compositions of HIPVs (Mumm and Dicke 2010). Quantitative and qualitative variations in the HIPV blends occur between healthy and herbivore-infested plants (Mumm and Dicke 2010; Danner et al. 2018), and these differences are known to induce different behavioural responses by parasitoid species to the HIPVs of host-infested plants (De Moraes et al. 1998; McCormick et al. 2012; Takabayashi and Shiojiri 2019).

Parasitoids also locate their hosts through kairomones emanating from the hosts or from the host by-products such as larval frass, larval secretions and adult sex pheromones (Vet and Dicke 1992; Afsheen et al. 2008). Herbivore larval frass volatiles mainly contain relatively small quantities of volatile compounds emitted by the host plants fed upon and other volatiles resulting from digestion and oxidation processes (Cordero et al. 2012), and may therefore only be active at short range, or act synergistically with HIPVs to enhance the long-range location of host-infested plants by natural enemies (Reddy et al. 2002; Dalen et al. 2015). Synergy between HIPVs and insect pheromones in enhancing mate and host finding behaviour by parasitoids has been documented (Reddy and Guerrero 2004; Xu et al. 2017). Herbivore pheromones are highly species-specific chemicals released for short and long-range communication with conspecific individuals, but which in turn can also be exploited by their natural enemies to locate them (Aukema and Raffa

2005); as seen in the braconid parasitoid *Chelonus insularis* Cresson, which is attracted to the sex pheromone of its host, *Spodoptera frugiperda* (Smith) (Roque-Romero et al. 2020).

The invasive South American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a serious constraint to tomato production both in open fields and in greenhouses (Desneux et al. 2010). Following its transatlantic invasion, *T. absoluta* was first detected in Spain in 2006 (Urbaneja et al. 2007). Soon after, the pest spread to and established in several countries in Europe, Asia, Haiti and almost the entire African continent with a devastating impact on tomato production (Desneux et al. 2010; Mansour et al. 2018; Verheggen & Fontus 2019; Han et al. 2019). Damage to crops by *T. absoluta* is mainly caused by the larvae which feed on leaves, fruits and stems of tomato plants, causing between 80 and 100% yield losses when no control measures are used (Desneux et al. 2010; Mansour et al. 2018 and references therein). Synthetic broad-spectrum chemical insecticides are often applied for *T. absoluta* control worldwide (Desneux et al. 2010; Biondi et al. 2018). These chemicals are, however, hazardous for human health and for the environment. The indiscriminate and frequent applications of these chemicals coupled with the multivoltine nature of *T. absoluta* have led to development of resistance in field populations of the pest to different classes of synthetic chemical insecticides, which inevitably makes their use unsustainable (reviewed in Guedes et al. 2019). Biological control using natural enemies offers an alternative and potentially sustainable way to control *T. absoluta* on tomato crops in the field (Desneux et al. 2010; Zappalà et al. 2013; Salas Gervassio et al. 2019).

The koinobiont endoparasitoid *Dolichogenidea gelechiidivoris* March (Syn. *Apanteles gelechiidivoris* March) (Hymenoptera: Braconidae) was imported into Kenya from Peru by the International Centre of Insect Physiology and Ecology (*icipe*) jointly with the International Potato Center (CIP) (Lima, Peru) for classical biological control of the pest in Africa. *Dolichogenidea gelechiidivoris* preferentially parasitises first and second instars of *T. absoluta* larvae (Aigbedion-Atalor et al. 2020), and it is known to be efficient in controlling *T. absoluta* in its native home range (Valencia and Peñaloza 1990; Palacios and Cisneros 1995; Vallejo 1999). However, when released for classical biological control, natural enemies are likely to emigrate or disperse from cropping field sites, thereby reducing their efficiency in controlling insect pests in the target crops (Heimpel and Asplen 2011). Interestingly, semiochemical-based lure applications have been pointed out as a sound solution to limit the dispersal of natural enemies and to recruit and retain them in the vicinity of target crops, thus enhancing biological control strategies (Kelly et al. 2014; Peri et al. 2018; Ayelo et al. 2021). A recent study reported that the egg parasitoid *Trichogramma achaeae* Nagaraja & Nagarkatti (Hymenoptera:

Trichogrammatidae) was attracted to *T. absoluta*-infested tomato plant volatiles and the host sex pheromone (Gontijo et al. 2019). We hypothesised that the larval parasitoid *D. gelechiidivoris* also exploits plant and host semiochemicals to locate *T. absoluta* in the field. We, therefore, assessed the attractiveness of the parasitoid to healthy and *T. absoluta*-infested tomato plant volatiles and host kairomones in relation to host densities, analysed the plant and host frass volatiles, and then determined the compounds attractive to the parasitoid. We discuss our findings in the light of developing a kairomone attractant-based lure to recruit and retain *D. gelechiidivoris* in tomato crop fields to enhance the biological control of *T. absoluta*.

Materials and methods

Plants

Tomato (*Solanum lycopersicum* L. (Solanaceae)) plants (cv. Kilele F1 hybrid, Syngenta) were grown and maintained in a screen house at 28 ± 5 °C, $60 \pm 10\%$ RH at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. Nurseries were established by sowing seeds on a mixture of 1:1 soil: manure (i.e. goat dung) in a seed raising plastic tray. Three weeks later, the seedlings were transplanted on a 3:1 soil: manure mixture in plastic pots (15×10 cm) at a density of one plant per pot. The plants were watered as needed, and provided weekly with water-dipped fertiliser containing 18% N, 20% P_2O_5, and 21% K_2O (Easygrow, Osho Chemical Industries Ltd). No chemical insecticides were applied on the plants.

Insects rearing

Tuta absoluta colony was established using eggs, larvae and adults obtained from infested tomato samples collected from fields at the Kenyan Agricultural and Livestock Research Organisation (KALRO) (0°37′11.3″S 37°22′08.0″E) in Kimbimbi, Mwea Sub-county, Kirinyaga County. The insects were kept in Plexiglass cages ($60 \times 60 \times 80$ cm) in the laboratory under conditions set at 25 ± 2 °C, $65 \pm 5\%$ RH and 12L:12 D photoperiod regime. To maintain the colony, 6–8-week-old tomato plants were exposed for 3 days to *T. absoluta* adults for oviposition. Thereafter, the infested plants were removed and placed in another Plexiglass cage and kept for 2 weeks albeit, with an addition of tomato plants to ensure better development of larvae. Emerging adults were transferred into another Plexiglass cage for plant infestation. *Tuta absoluta* adults were fed on 80% honey solution smeared on the top inner side of the Plexiglass cages.

Dolichogenidea gelechiidivoris colony was initiated with parasitoid cocoons received from the International Potato Center (CIP), Peru and maintained at the *icipe* quarantine facility until the parasitoid adults emerged, as described in Aigbedion-Atalor et al. (2020). The rearing was done in laboratory Plexiglas cages ($40 \times 40 \times 50$ cm) at the quarantine facility at 25 ± 2 °C, $65 \pm 5\%$ RH and photoperiod regime of 12L:12D. The insects were kept on *T. absoluta*-infested tomato plants obtained by exposure of 6–8-week-old tomato plants to *T. absoluta* adults for 3 days. Thereafter, at three to 5 days post-infestation, the infested plants with batches of first and second larval instars were offered to the parasitoids for 48 h and renewed on 2-day intervals. Parasitoid-exposed plants were cut off and their petioles inserted into floral foams previously soaked into water, and then placed in other Plexiglass cages ($30 \times 30 \times 30$ cm). Healthy tomato plants were added to ensure satisfactory larval development. Emerging *D. gelechiidivoris* adults were aspirated and kept in small cylindrical plastic cups (2.5×7 cm) for 48 h to increase the chances of their mating before they were used in bioassay experiments. Parasitoids were fed on 80% honey solution smeared on the inner side top of the Plexiglass cages.

Y-tube olfactometer bioassays

Dolichogenidea gelechiidivoris responses to volatiles from different odour sources were tested in dual-choice experiments using a Y-tube olfactometer. The Y-tube glass devise (2.5 cm i.d.) consisted of a 12-cm long stem and two 6-cm arms which formed an angle of 60º. The Y-tube glass device was placed in an observation chamber made of a cardboard box ($35 \times 35 \times 55$ cm), which was illuminated with a 220–240 V cool fluorescent tube to provide a uniform lighting. Each arm of the olfactometer was connected to a 10-L glass jar serving as an odour source container. A vacuum pump (KNF lab LABOPORT N86KT.18, France) was used to generate a unidirectional airflow which was filtered by an active carbon filter, and then passed through the odour source containers at a constant wind speed of 150 mL min^{-1} set using a flow meter (AALBORG, Orangeburg, NY, USA). Although preliminary bioassays showed that the proportion of responsive parasitoids was quite similar when the Y-tube glass was oriented vertically (96.7%) or horizontally (90%), in subsequent bioassays, the Y-tube glass was oriented vertically in our tests, because the parasitoids move upwards in rearing cages. The plant pot was tightly wrapped with aluminium foil to prevent headspace volatile contamination from the soil. The test plants were then placed in the glass jars and left to acclimatise under the airflow for one hour before the start of the bioassays. A single parasitoid was introduced at the base of the stem of the Y-tube,

and the insect's first choice was recorded over a 10 min observation period. A choice was scored when the insect climbed and entered a given arm up to a distance of 3 cm and remained in the arm for 30 s. In total, fifty insects were tested per choice test over 10 days (five insects per day per choice test). Insects that had not made a choice after 5 min were considered as non-responsive (which accounted for between 0 and 6% of the insects tested), and hence were not included in the data analyses. One plant was used for five insects, and after three insects the Y-tube glass was cleaned with dichloromethane (solvent) and the volatile sources switched between arms to avoid positional bias. At the end of experiments on each day, the Y-tube glass was cleaned with Teepol odourless detergent and hot water, and rinsed with acetone and distilled water, then dried in the oven at 150 °C overnight. Naïve 2–5-day-old *D. gelechiidivoris* females were used in the bioassays as the parasitoid's mature egg load peaked within this age interval (Aigbedion-Atalor et al. 2020). All bioassays were performed at the quarantine facility at 25 ± 2 °C, $65 \pm 5\%$ RH and 12L:12D photoperiod regime.

Responses of *Dolichogenidea gelechiidivoris* to plant volatiles

The olfactory responses of *D. gelechiidivoris* to volatiles from healthy and *T. absoluta*-infested tomato plants were investigated in dual choices, comparing: (i) air vs. air (control); (ii) air vs. healthy plant; (iii) air vs. *T. absoluta*-infested plant; (iv) healthy plant vs. *T. absoluta*-infested plant. Four-week-old plants were individually infested with 5, 10 or 20 T. *absoluta* larvae of first and second instars (1:1 ratio) which were left to feed on the plant for 4 days.

Responses of *Dolichogenidea gelechiidivoris* to host volatiles

The parasitoid olfactory responses to volatiles from *T. absoluta* larvae, larval frass and sex pheromone were tested; here the 10-L glass jar containers were replaced by 250-mL quick fit glass jars (Sigma Scientific, Gainesville, FL, USA). For the bioassays, the clean air (control) was tested against: (i) clean air; (ii) 10, 20 or 40 T. *absoluta* larvae; (iii) 15, 30 or 60 mg of *T. absoluta* larval frass; and (iv) *T. absoluta* female commercial sex-pheromone lure. The pheromones lures contain 90:10 ratio of (*E,Z,Z*)-3,8,11-tetradecatrien-1-yl acetate to (*E,Z*)-3,8-tetradecadien-1-yl acetate (both 98.5% purity) released by a rubber septum dispenser and were purchased from KOPPERT (Biological Systems, Ltd.). *Tuta absoluta* larval frass was collected from mined tomato plant leaves using a camel hairbrush, and thereafter immediately used in the bioassays. A sample of 60 mg of frass corresponded to the amount collected from approximately 40 third instar

larvae feeding for 4 days. The collection of larvae was done by placing *T. absoluta* larvae-infested leaves in a plastic box, then paper towel was added and the container was closed with a ventilated-mesh lid. After 24 h, the larvae easily moved out of the mined leaves, which made easy the collection of the larvae without damaging them. *Tuta absoluta* first and second larval instars were used in the bioassays as the parasitoid *D. gelechiidivoris* preferentially parasitises these stages (Aigbedion-Atalor et al. 2020).

Collection of volatiles

Tomato plant headspace volatiles

Headspace volatiles were trapped onto prepacked 30-mg Super-Q adsorbents (Analytical Research Systems, Gainesville, FL, USA) using a dynamic push–pull system. A vacuum pump (KNF lab LABOPORT N86KT.18, France) was used to suck air which passed through a charcoal-filter and then entered the 10-L glass jar containers used in the behavioural experiments, at a rate of 200 mL min^{-1}. We collected volatile compounds from healthy and *T. absoluta*-infested tomato plants, as well as from empty volatile collection chamber, and from pots containing soil with no plants (controls), with five replicates. The pots of the plant and the control were tightly wrapped in aluminium foil to prevent contamination from potting soil, and were then placed separately in the containers. The age of tomato plants and the infestation procedure were the same as described above. Volatiles were collected for 24 h and eluted with 150 µL of dichloromethane, then stored at -80 °C. Just before analyses by gas chromatography-mass spectrometry (GC–MS), the headspace extract volume was concentrated to 50 µL under a gentle nitrogen air stream, then 5 µL of biphenyl (99% purity) solution (20 ng/µL) was added as internal standard.

Frass headspace volatiles

A Solid Phase Micro-Extraction (SPME) holding 65-µm fibre (PDMS-DVB StableFlex; Supelco Bellefonte, PA, USA) was used to collect *T. absoluta* larval frass volatiles. The SPME fibre was cleaned by conditioning in GC at 250 °C for 15 min before use. A 60 mg sample of larval frass was placed in a 2-mL storage vial with a rubber septum lid. The SPME fibre was deployed through the lid and held at 1–2 cm above the sample for 24 h, thereafter the volatiles were analysed by GC–MS, in five replicates.

Chemical analysis

Volatile analyses were performed using a 7890A Gas Chromatograph (Agilent Technologies) coupled with an HP-5MSI low bleed non-polar capillary column (5% phenyl and

95% methylpolysiloxane, 30 cm×0.25 mm×0.25 μm film thickness). An aliquot (1 μL) of headspace tomato volatiles was analysed in splitless mode. Whereas the SPME fibre loaded with the frass volatiles was injected into the GC injection port immediately after volatile trapping. Volatiles were analysed using helium as carrier gas at a flow rate of 1.2 mL min^{-1}. For both the plant and frass volatile analyses, the oven temperature was set at 35 °C for 5 min, then increased at a rate of 10 °C min^{-1} to reach 280 °C which was held for 10.5 min. The ion source temperature was set at 250 °C with an interface temperature of 270 °C, and spectra were recorded at 70 eV. The compounds were identified using retention time, library mass spectra (NIST11, Wiley9, Adams and Chemecol), and Kovats retention indices (RIs). The RIs calculated using retention times of n-alkane (C_8–C_{30}) standards which were run as a mixture in a separate injection. The retention index (RI) was computed using the following formula: $(RI) = [RT(X) - RT(n)]/[RT(n+1) - RT(n)]*100 + (100*n)$, where RT(X) is the retention time of the studied compound X, RT(n) is the retention time of the alkane with n carbons that eluted before X, and RT$(n+1)$ is the retention time of the alkane with $n+1$ carbons that eluted after X. Comparison with published mass spectra and Kovats retention indices from online NIST library was done, and synthetic standards, when available, were run to confirm the identification of some compounds by comparison of the expected retention time and the MS spectra. The peak area and concentration of the internal standard were used for quantification of the volatile organic compounds (VOCs), i.e. internal calibration which is a recommended method for quantification of relative amounts of volatile compounds using GC–MS (IOFI 2011; Ruiz-Hernández et al. 2018). In our study, the formula used for the quantification of the compounds were adapted from Wang et al. (2019), as follows:

$$Ca = \frac{\left(\frac{PAa}{PAis}\right) x \; Cis \; x \; 5\mu L}{V}$$

and

$$Rr = \frac{Ca \; x \; V}{24h}$$

where Ca is the concentration (ng/μL) of the analyte in the volatile eluent; PAa is the peak area of the identified analyte; PAis is the peak area of the internal standard; Cis is the concentration of internal standard (i.e. 20 ng/μL of biphenyl) and V is the volume of the volatile eluent (i.e. 50 μL) in which the aliquot (5μL) of internal standard has been applied; Rr is the release rate (ng/plant/h) which equals to the concentration (ng/μL) multiplied by the volume of the eluent (μL) and divided by the volatile collection period (24 h).

Chemicals

All synthetic standards were purchased from Merck, France. Chemical purities of standards were as follows: α-pinene (98%), sabinene (75%), β-myrcene (90%), 2-carene (97%), α-phellandrene (85%), 3-carene (90%), α-terpinene (95%), β-phellandrene (95%), β-ocimene (90%), linalool (97%), allo-ocimene (98%), methyl salicylate (97%), (E)-β-caryophyllene (98%), α-humulene (96%), p-cymene (99%), p-xylene (99%), α-terpineol (96%), terpinolene (90%), γ-terpinene (97%), γ-elemene (98%), β-elemene (96%), (Z)-3-hexen-1-ol (98%), 6-methyl-5-hepten-2-one (98%), geranyl acetone (97%), β-pinene (99%) and β-ionone (96%). The synthetic of β-ocimene contained the mixture of the isomers E and Z in a ratio of 2.5E: 1Z. Dichloromethane (99.9% purity) was purchased from Merck, Germany.

Bioassays with synthetic compounds

The VOCs α-pinene, sabinene, β-pinene, β-myrcene, 2-carene, α-phellandrene, 3-carene, α-terpinene, β-phellandrene, (Z)-β-ocimene, (E)-β-ocimene, linalool, allo-ocimene, methyl salicylate, (E)-β-caryophyllene and α-humulene were selected to test the attractiveness of synthetic compounds to the parasitoid. The synthetic of β-ocimene containing the mixture of E and Z isomers in a ratio of 2.5E: 1Z, was tested instead of the two isomers separately. The natural release rate (ng/plant/h) (Table 1) was considered as the reference dose, and each compound was tested at three doses corresponding to release rates of 1, 10 and 100 equivalent plants. Based on the results, seven compounds (i.e. α-pinene, β-myrcene, α-phellandrene, α-terpinene, β-ocimene, methyl salicylate and (E)-β-caryophyllene) were found to be attractive to the parasitoid, and therefore a 7-component blend of these compounds mixed at their most attractive doses (blend B1) was tested. Since this dose of blend B1 did not attract the parasitoid, four other blends were made up from blend B1 dose by dilutions to one-half (blend B2), one-fourth (blend B3), one-tenth (blend B4), and one-hundredth (blend B5), with which the attractiveness to the parasitoid was tested. The compounds were diluted in dichloromethane (solvent), and a 10 μL aliquot of the test solution was loaded on a 2×2 cm filter paper and tested against the control (filter paper loaded with 10 μL-dichloromethane). The solvent was left to evaporate for 30 s, then the impregnated filter papers were placed at the edge of the olfactometer arms, and were renewed for every insect. Fifty insects were tested individually per choice test as described above.

Table 1 Mean amount (ng/plant/h) of volatile organic compounds identified in the headspace of healthy plants and *Tuta absoluta* larvae-infested tomato plants (n = 5)

Peak No.[a]	RT (min)	KI[b]alk	KI[c]lit	Compound[d]	Chemical class	Healthy plants	Plants infested with 5, 10 and 20 T. *absoluta* larvae			P value[e]
							5	10	20	
1	8.06	861	858	(Z)-3-hexenol*	Alcohol	nd	nd	0.28 ± 0.15^a	1.37 ± 0.32^b	0.002
2	8.32	870	865	p-xylene*	Benzenoid	0.05 ± 0.01^a	0.22 ± 0.04^b	0.37 ± 0.11^b	1.02 ± 0.55^b	0.005
3	9.83	936	934	α-pinene*	Monoterpene	1.68 ± 0.22^a	3.59 ± 0.22^b	6.73 ± 0.45^b	12.26 ± 1.95^c	<0.001
4	10.42	963	963	benzaldehyde	Aldehyde	nd	0.08 ± 0.05^a	0.52 ± 0.15^{ab}	0.95 ± 0.12^b	0.002
5	10.62	972	972	3,7,7-trimethyl-1,3,5-cycloheptatriene	Homoterpene	2.64 ± 0.49^a	4.56 ± 0.34^{ab}	9.33 ± 0.91^{bc}	16.43 ± 2.57^c	0.001
6	10.68	975	975	sabinene*	Monoterpene	nd	0.24 ± 0.02^a	1.03 ± 0.16^b	1.71 ± 0.43^c	0.001
7	10.73	977	981	β-pinene*	Monoterpene	0.11 ± 0.02^a	0.26 ± 0.01^{ab}	0.50 ± 0.11^{bc}	0.70 ± 0.07^c	0.001
8	10.78	980	983	trans-isolimonene	Monoterpene	0.27 ± 0.11^a	0.22 ± 0.12^a	1.24 ± 0.41^{bc}	3.06 ± 0.68^c	0.003
9	10.99	989	987	6-methyl-5-hepten-2-one *	Ketone	nd	0.18 ± 0.08^a	0.31 ± 0.07^{ab}	1.34 ± 0.54^b	0.017
10	11.08	994	992	β-myrcene*	Monoterpene	0.49 ± 0.15^a	0.98 ± 0.07^{ab}	2.43 ± 0.32^{bc}	5.85 ± 0.48^{bc}	0.001
11	11.25	1002	1001	2-carene*	Monoterpene	15.40 ± 1.0^a	30.01 ± 1.84^{ab}	43.96 ± 3.91^{bc}	73.52 ± 6.88^c	<0.001
12	11.32	1006	1005	α-phellandrene*	Monoterpene	2.99 ± 0.21^a	5.54 ± 0.40^{ab}	$12.38\pm0.75b^c$	15.42 ± 1.60^c	0.001
13	11.41	1011	1011	3-carene*	Monoterpene	0.17 ± 0.07^a	0.70 ± 0.09^b	0.97 ± 0.05^b	2.17 ± 0.33^c	0.003
14	11.52	1017	1018	α-terpinene*	Monoterpene	1.40 ± 0.11^a	2.43 ± 0.28^{ab}	4.71 ± 0.49^{bc}	8.53 ± 1.85^c	0.001
15	11.69	1027	1026	p-cymene*	Monoterpene	0.79 ± 0.16^a	1.59 ± 0.22^b	1.93 ± 0.38^b	1.04 ± 0.19^{ab}	0.024
16	11.80	1033	1032	β-phellandrene*	Monoterpene	42.24 ± 2.24^a	63.70 ± 3.36^{ab}	92.07 ± 10.07^{bc}	142.06 ± 8.17^c	<0.001
17	11.93	1040	1039	(Z)-β-ocimene*	Monoterpene	0.06 ± 0.02^a	0.19 ± 0.04^{ab}	0.42 ± 0.03^{bc}	1.18 ± 0.48^c	<0.001
18	12.03	1046	1044	benzene acetaldehyde	Aldehyde	nd	0.40 ± 0.25	0.34 ± 0.14	0.74 ± 0.21	0.057
19	12.11	1050	1050	(E)-β-ocimene*	Monoterpene	0.23 ± 0.06^a	0.65 ± 0.05^{ab}	1.14 ± 0.09^{bc}	3.20 ± 0.27^c	<0.001
20	12.31	1061	1060	γ-terpinene*	Monoterpene	0.54 ± 0.19^a	1.00 ± 0.23^a	1.21 ± 0.49^{ab}	2.14 ± 0.37^b	0.047
21	12.83	1090	1091	terpinolene*	Monoterpene	0.44 ± 0.06^a	$0.80\pm0.06a^b$	$1.19\pm0.31b^c$	2.59 ± 0.52^c	0.004
22	13.03	1100	1101	linalool*	Monoterpene	nd	nd	$0.06\pm0.02a$	$0.50\pm0.08b$	0.001
23	13.41	1122	1128	allo-ocimene*	Monoterpene	nd	0.24 ± 0.04^a	0.27 ± 0.08^a	0.52 ± 0.15^b	0.006
24	13.72	1139	1142	neo-allo ocimene	Monoterpene	nd	nd	0.04 ± 0.02^a	0.21 ± 0.08^b	0.016
25	14.30	1171	–	unidentified	Monoterpene	0.06 ± 0.03	0.12 ± 0.05	0.12 ± 0.06	0.26 ± 0.11	0.491
26	14.39	1176	1182	(Z)-3-hexenyl butanoate	Ester	nd	nd	0.09 ± 0.06^a	0.47 ± 0.07^b	0.011
27	14.45	1180	1183	dill ether	Monoterpene	0.05 ± 0.02^a	0.36 ± 0.09^a	0.50 ± 0.09^{ab}	0.77 ± 0.12^b	0.003
28	14.55	1190	1193	α-terpineol*	Monoterpene	nd	nd	0.07 ± 0.03	0.09 ± 0.04	0.348
29	14.62	1201	1197	methyl salicylate*	Benzenoid ester	nd	nd	0.91 ± 0.11^a	2.61 ± 0.18^b	0.001
30	14.94	1220	1218	β-cyclocitral	Monoterpene	nd	nd	nd	0.15 ± 0.07	–
31	16.68	1340	1342	δ-elemene	Sesquiterpene	0.42 ± 0.15^a	0.33 ± 0.04^a	1.35 ± 0.54^{ab}	2.23 ± 0.61^b	0.013
32	17.40	1388	1385	β-elemene*	Sesquiterpene	nd	nd	0.58 ± 0.19^a	1.31 ± 0.22^b	0.001
33	17.47	1404	1406	(Z)-jasmone	Ketone	nd	nd	0.04 ± 0.02^a	0.33 ± 0.16^b	0.048
34	17.72	1424	1422	α-cedrene	Sesquiterpene	0.04 ± 0.01^a	0.06 ± 0.01^a	0.15 ± 0.07^{ab}	0.36 ± 0.09^b	0.013
35	17.80	1430	1427	(E)-β-caryophyllene*	Sesquiterpene	0.91 ± 0.80^a	$1.67\pm.23^{ab}$	4.92 ± 0.88^b	9.95 ± 0.85^c	<0.001
36	17.92	1442	1440	γ-elemene*	Sesquiterpene	0.03 ± 0.01^a	0.12 ± 0.05^{ab}	0.22 ± 0.07^{bc}	0.49 ± 0.04^c	0.004

Table 1 (continued)

Peak No.[a]	RT (min)	KI[b]alk	KI[c]lit	Compound[d]	Chemical class	Healthy plants	Plants infested with 5, 10 and 20 T. absoluta larvae			P value[e]
							5	10	20	
37	18.04	1450	1444	guaiadiene-6,9	Sesquiterpene	0.07 ± 0.02	0.13 ± 0.7	0.25 ± 0.07	0.32 ± 0.16	0.424
38	18.10	1455	1454	geranyl acetone*	Ketone	0.04 ± 0.02^a	0.46 ± 0.06^b	0.61 ± 0.11^b	1.72 ± 0.53^b	0.004
39	18.24	1466	1462	α-humulene*	Sesquiterpene	0.23 ± 0.04^a	0.58 ± 0.11^a	1.43 ± 0.27^{ab}	2.72 ± 0.14^b	<0.001
40	18.64	1490	1488	β-ionone*	Ketone	nd	nd	0.22 ± 0.13	1.15 ± 0.58	0.059
Total VOCs						71.74 ± 3.88^a	121.40 ± 4.70^{ab}	194.87 ± 12.59^{bc}	323.41 ± 20.08^c	<0.001
Monoterpenes						67.31 ± 3.59^a	112.61 ± 4.86^{ab}	173.04 ± 0.56^{bc}	278.39 ± 18.71^c	<0.001
Sesquiterpenes						1.70 ± 0.25^a	2.89 ± 0.46^{ab}	8.90 ± 1.61^{bc}	17.37 ± 0.60^c	0.001

[a]Peak numbers correspond to peaks indicated in Fig. 3

[b]Retention index relative to C_8–C_{30} n-alkanes of a HP-5MS capillary column

[c]Retention index obtained from the literature (e.g. Khan et al. 2012)

[d]Identification of compounds based on the retention time (RT), retention indices and mass spectra using libraries, i.e. NIST11 and Wiley9, Adams and Chemecol, and comparison with published mass spectra and retention indices from online NIST library. *indicates compounds confirmed with authentic standards

[e]p value of the nonparametric Kruskal–Wallis ANOVA for comparison of volatile compounds between healthy and T. absoluta-infested plants. Significant values are in bold
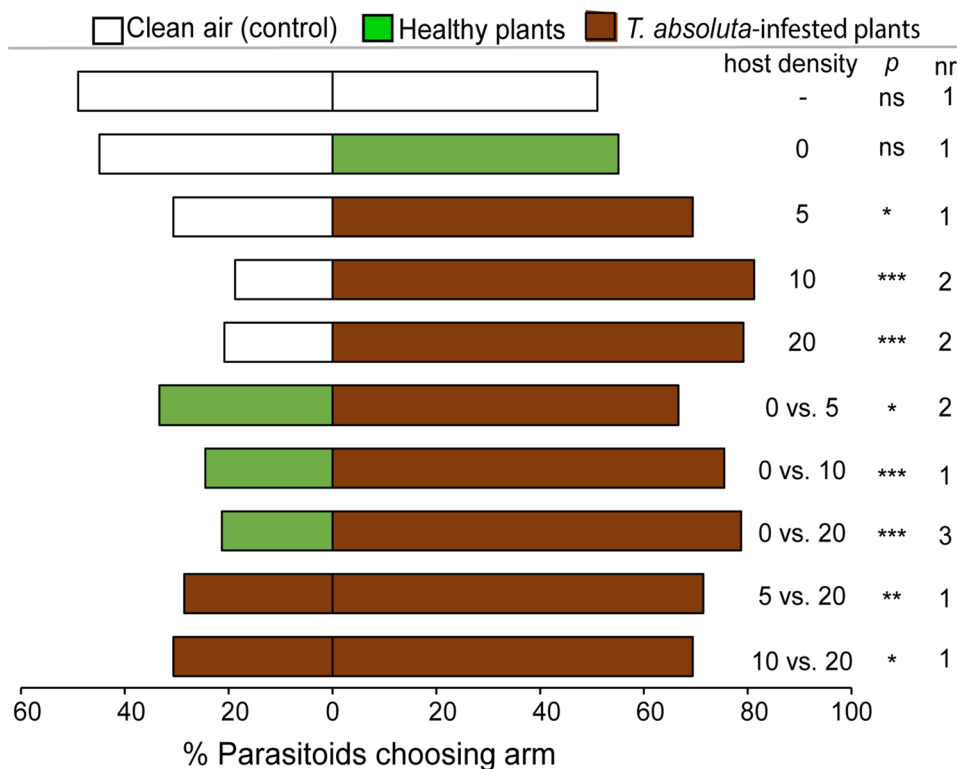
nd = not detected

Statistical analyses

The proportions of odours chosen by the parasitoid *D. gelechiidivoris* were compared using a chi-squared test. The variable VOC concentrations were tested for normality using Shapiro–Wilk's test, and homogeneity of variance using Bartlett's test. Since the data were not normally distributed and their variance not homogenous, a nonparametric Kruskal–Wallis ANOVA was performed to compare amounts of VOCs between healthy and *T. absoluta*-infested tomato plants, using the post hoc Dunn's test with Bonferroni's adjustment for mean separation (Dinno 2015). The random forest (RF) analysis (Breiman 2001) was performed to select the VOCs that best distinguished *T. absoluta*-infested tomato plants from healthy plants based on the concentrations of VOCs. The mean decrease in accuracy (MDA) generated using the RF "importance" function enabled the selection of VOCs that significantly contributed to the discrimination analyses, and a higher MDA indicates a higher importance of the compound to the discrimination (Liaw and Wiener 2002; Ranganathan and Borges 2010). A classification error, commonly known as out of bag (OOB) error, allowed appreciating the classification accuracy (100%—OOB error) of the RF analysis output (Breiman 2001). Using concentrations of the most discriminating VOCs, a sparse partial least square discriminant analysis (sPLS-DA) biplot was performed in mixOmics package to illustrate the correlation between volatile compounds and healthy and *T. absoluta*-infested plants (Lê Cao et al. 2011; Hervé et al. 2018). The validation of the sPLS-DA model was assessed using the "perf" function and the "leave-one-group-out" cross-validation method in the mixOmics package, as well as the sPLS-DA parameters (R2X, R2Y, Q2) (Rohart et al. 2017). Heatmap clustering was also performed to illustrate variations in the discriminating VOCs across replicates of healthy and *T. absoluta*-infested tomato plants, using the "cim" function in the mixOmics package (Rohart et al. 2017). For bioassays with synthetic compounds, we used compounds that appeared at least thrice among the top discriminating VOCs (compounds indicated in dark black colour) responsible for differences between plants offered in dual-choice bioassays in the five modalities in which the parasitoid attraction was significant. All statistical analyses were performed using R statistical software, version 4.0.2 (R Core Team 2020).

Results

Responses of *Dolichogenidea gelechiidivoris* to plant volatiles

The parasitoid *D. gelechiidivoris* was not attracted to volatiles of healthy tomato plants when compared to clean air ($\chi^2 = 0$, df = 1, $P = 1$) (Fig. 1). In contrast, relative to clean air, the parasitoid was highly attracted to volatiles of tomato plants infested by *T. absoluta* at all the densities of host infestation tested ($\chi^2 = 6.61$, df = 1, $P = 0.01$; $\chi^2 = 17.52$, df = 1, $P < 0.0001$; $\chi^2 = 15.19$, df = 1, $P < 0.0001$, for 5, 10 and 20 *T. absoluta* larvae/plant). Similarly, *D. gelechiidivoris* preferred the volatiles of tomato plants infested with 5 ($\chi^2 = 4.69$, df = 1, $P = 0.03$), 10 ($\chi^2 = 10.58$, df = 1, $P = 0.0006$) or 20 *T. absoluta* larvae ($\chi^2 = 14.38$, df = 1, $P = 0.0001$) over those of healthy tomato plants (Fig. 1). Moreover, the parasitoid was more attracted to volatiles of tomato plants infested with a high density of 20 *T. absoluta* larvae than to the volatiles of plants with low infestation levels, i.e. 5 ($\chi^2 = 8.16$, df = 1, $P = 0.004$) or 10 *T. absoluta* larvae ($\chi^2 = 6.61$, df = 1, $P = 0.01$) (Fig. 1).

Responses of *Dolichogenidea gelechiidivoris* to host volatiles

Volatiles of *Tuta absoluta* larvae did not attract the parasitoid, *D. gelechiidivoris*, at any of the tested densities of 10 ($\chi^2 = 0.02$, df = 1, $P = 0.887$), 20 ($\chi^2 = 0.73$, df = 1, $P = 0.391$) or 40 larvae ($\chi^2 = 3.38$, df = 1, $P = 0.065$) compared to clean air (Fig. 2). Similarly, the parasitoid was not attracted to volatiles from *T. absoluta* larval frass at the doses of 15 mg ($\chi^2 = 0.98$, df = 1, $P = 0.322$) or 30 mg ($\chi^2 = 2.04$, df = 1, $P = 0.153$) when compared to clean air. On the other hand, volatiles of *T. absoluta* larval frass at a dose of 60 mg did attract the parasitoid compared to clean air ($\chi^2 = 8.16$, df = 1, $P = 0.004$) (Fig. 2). Moreover, *D. gelechiidivoris* was more attracted to the commercial sex pheromone of *T. absoluta* than to clean air ($\chi^2 = 6.02$, df = 1, $P = 0.014$) (Fig. 2).

Analysis of tomato volatiles

Forty VOCs were identified in the headspace volatile profiles of healthy and *T. absoluta* larvae-infested tomato plants (Table 1; Fig. 3). These VOCs belong to eight chemical classes: monoterpenes (22), sesquiterpenes (7), ketones (4), aldehydes (2), benzenoids (2), an ester (1), a homoterpene (1) and an alcohol (1) (Table 1). Quantitative and qualitative differences were observed in the composition of constitutive and *T. absoluta* larvae-induced tomato plant volatiles which were dominated by monoterpenes and

Fig. 1 Behavioural responses of *Dolichogenidea gelechiidivoris* to volatiles of *Tuta absoluta* larvae-infested and healthy tomato plants in Y-tube olfactometer choice tests, as a percentage of the parasitoids which responded. Fifty insects were tested per choice test. n_r = number of non-responsive parasitoids (i.e. insects which made no choice). *P* stands for levels of significance with n_s = no significant difference ($P > 0.05$); *, **, *** = significant differences, respectively, at $P < 0.05$, $P < 0.01$ and $P < 0.001$ from χ^2 test at $\alpha = 0.05$

sesquiterpenes (Table 1; Fig. 3). Monoterpenes increased by twofold to fourfold in *T. absoluta* larvae-infested tomato plants relative to healthy plants. Relative to the total volatile emission per plant, monoterpenes accounted for 93.8, 92.8, 88.8 and 86.1%, respectively, for healthy plants and plants infested with 5, 10 and 20 T. *absoluta* larvae; 2-carene and β-phellandrene being the most abundant monoterpenes (Table 1). Sesquiterpenes increased by twofold to tenfold in *T. absoluta* larvae-infested plants compared to healthy plants, and represented 2.4, 2.4, 4.7 and 5.8% of the total released volatiles, respectively, in healthy plants, and 5, 10 and 20 T. *absoluta* larvae-infested plants, with (*E*)-β-caryophyllene, α-humulene and δ-elemene as the most abundant sesquiterpenes (Table 1). Specifically, VOC emission rates increased with the host infestation density, as seen in the emission of α-pinene, β-myrcene, 3,7,7-trimethyl-1,3,5-cycloheptatriene, 2-carene, α-phellandrene, α-terpinene, β-phellandrene, (*E*)-β-ocimene, (*E*)-β-caryophyllene, δ-elemene and α-humulene which increased in *T. absoluta* larvae-infested plants compared to healthy plants. Fifteen VOCs were specific to *T. absoluta*-infested tomato plant volatiles, and not detected among the volatiles of healthy plants. These VOCs included the alcohol (*Z*)-3-hexenol; the aldehydes benzaldehyde and benzene acetaldehyde; the monoterpenes sabinene, linalool, allo-ocimene, neo-allo ocimene, α-terpineol, β-cyclocitral; the sesquiterpene β-elemene; the ester (*Z*)-3-hexenyl butanoate; the benzenoid ester methyl salicylate; the ketones 6-methyl-5-hepten-2-one, (*Z*)-jasmone and β-ionone (Table 1). On the other hand, emission rates of some VOCs including γ-terpinene, guaiadiene-6,9 and an unidentified monoterpene did not significantly vary between healthy and *T. absoluta* larvae-infested tomato plants (Table 1).

Analysis of *Tuta absoluta* larval frass volatiles

Fifteen VOCs were detected in the headspace of *T. absoluta* larval frass volatiles, of which 7 were monoterpenes, 6 sesquiterpenes, a ketone and a benzenoid ester (Table 2, Fig. 4). With the exception of caryophyllene oxide, all VOCs detected in the larval frass were found in the tomato plant headspace volatiles listed in Table 1.

Determination of discriminating volatile organic compounds

The mean decrease in accuracy (MDA) in the random forest (RF) analysis revealed 19 VOCs as the most discriminating compounds (MDA ≥ 60) between healthy plants and plants infested with 5, 10 and 20 T. *absoluta* larvae (Fig. 5a). The sparse partial least square discriminant analysis (sPLS-DA) showed the distribution of the different plant treatments in four clusters, where plants infested by 5, 10 and 20 T. *absoluta* larvae were separated from each other, and from the control, i.e. healthy plants (Fig. 5b). The sPLS-DA biplot showed that all the discriminating VOCs were closely associated with 20 T. *absoluta* larvae-infested tomato plants, the volatiles of which elicited the greatest attraction in the parasitoid (Fig. 5c). A proportion of 83.3% of the total variation in volatile emission was explained by the two first dimensions of the sPLS-DA. Dimension 1 accounted for 76.7% of the total variation and was highly correlated mainly with methyl salicylate, (*E*)-β-ocimene, β-myrcene,

Fig. 2 Behavioural responses of *Dolichogenidea gelechiidivoris* to volatiles of larvae, larval frass, and commercial sex pheromone of *Tuta absoluta* in a Y-tube olfactometer choice tests, as a percentage of respondent parasitoids. Fifty insects were tested per choice test. n_r = number of non-responsive parasitoids (i.e. insects which made no choice). cph = one rubber septum loaded with commercial sex pheromone of *T. absoluta*. *P* stands for levels of significance with n_s = no significant difference (*P* > 0.05); *, ** = significant differences, respectively, at *P* < 0.05 and *P* < 0.01 from χ^2 test at α = 0.05
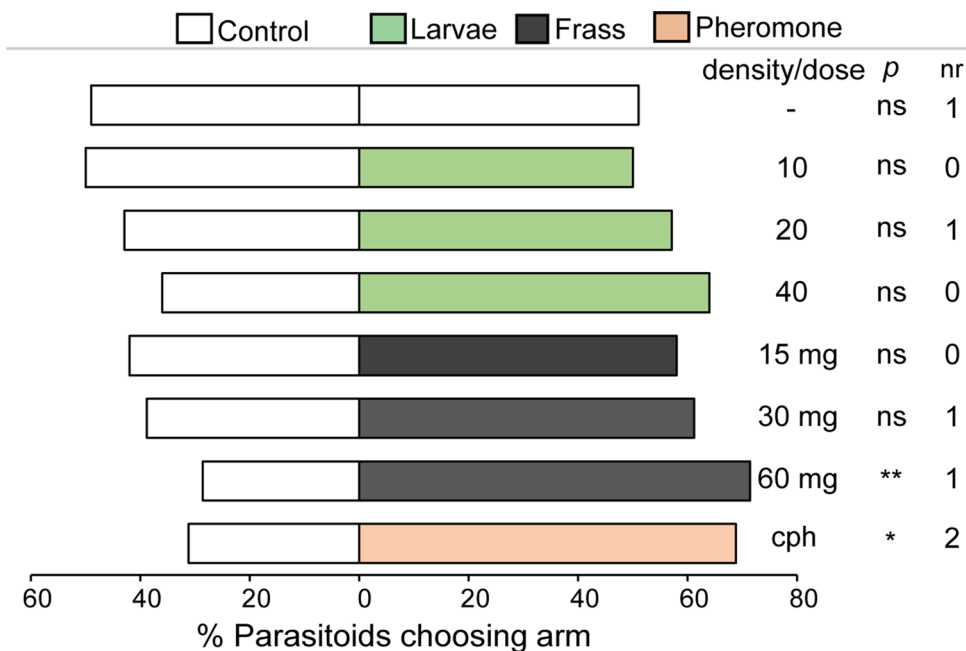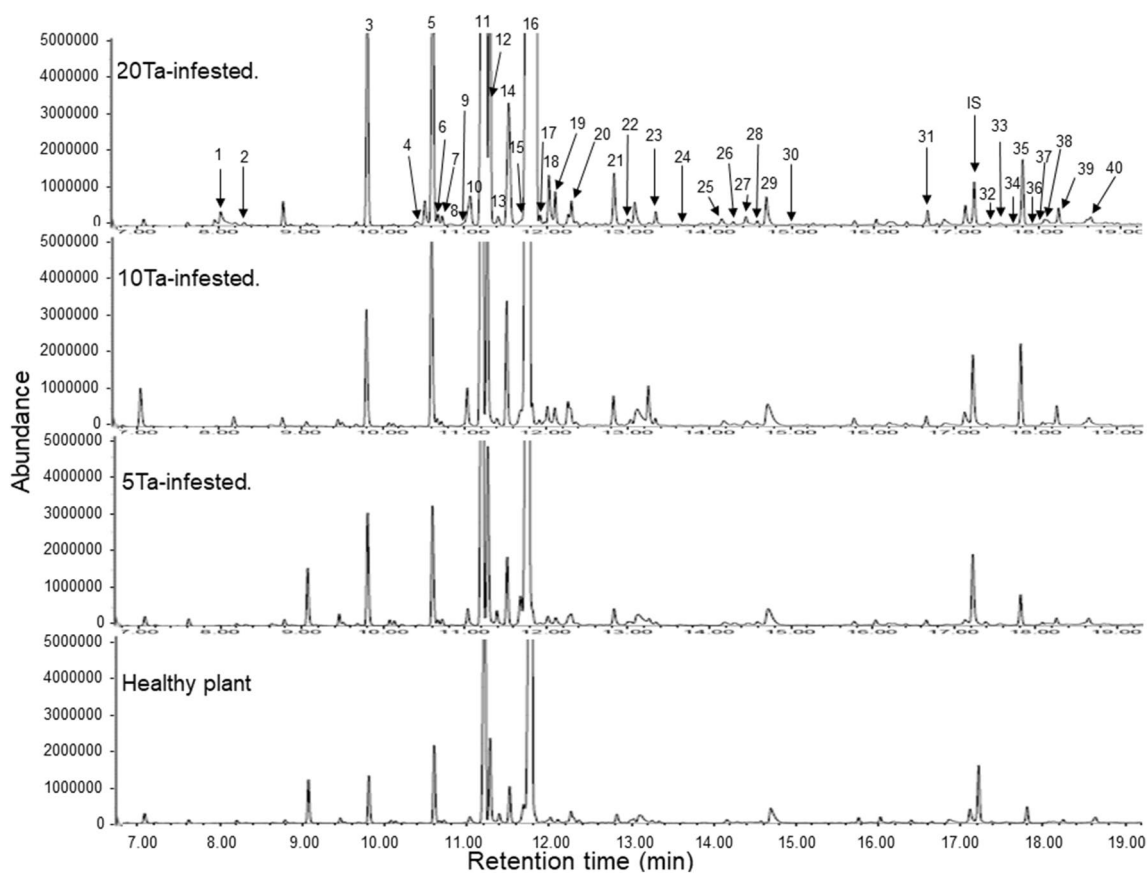
Fig. 3 GC/MS profiles of headspace volatiles from healthy plants and plants infested with 5, 10 and 20 *Tuta absoluta* (Ta) larvae. The numbers correspond to the names of the volatile compounds listed in Table 1. IS = internal standard (biphenyl)

β-phellandrene, *α*-humulene, (*E*)-*β*-caryophyllene, 2-carene and *α*-pinene. Whereas dimension 2 explained 6.6% of the total variation, and was closely associated mainly with linalool, 3-carene, *α*-phellandrene, *β*-pinene and allo-ocimene. Clustering heatmap also showed these VOCs to be abundant in tomato plants infested with 20 T. *absoluta* larvae (Fig. 5d). Moreover, the heatmap clustered the samples in two main categories, i.e. one composed of the plants infested with 20 T. *absoluta* larvae, and another group containing all the remaining samples (i.e. combination of healthy plants and others infested with 5 and 10 T. *absoluta* larvae) which, in turn, were also separated among themselves.

Using the RF analysis, we went further in details by highlighting the VOCs that mostly discriminated plants offered in dual choices where the parasitoid displayed attraction responses. The findings revealed that the top VOCs discriminating *T. absoluta* larvae-infested plants from healthy plants resulted mainly from quantitative variations, and included α-pinene, sabinene, *β*-pinene, 2-carene, *α*-phellandrene, 3-carene, *α*-terpinene, *β*-phellandrene, (*Z*)-*β*-ocimene, (*E*)-*β*-ocimene, (*E*)-*β*-caryophyllene, *α*-humulene, *β*-myrcene, allo-ocimene and geranyl acetone, with the exception of

methyl salicylate which was not detected in healthy plants (Fig. 6a–c). Similarly, *α*-pinene, *β*-myrcene, 2-carene, *β*-phellandrene, (*Z*)-*β*-ocimene, (*E*)-*β*-ocimene, (*E*)-*β*-caryophyllene and linalool appeared to be the VOCs that contributed most to distinguishing between plants with high infestation density, i.e. 20 T. *absoluta* larvae and those with low infestation density, i.e. 5 and 10 T. *absoluta* larvae (Fig. 6d–e). The multidimensional scaling (MSD) ordination plot also showed that healthy and infested plants differed in the abundance of their VOCs (Fig. 6f).

The compounds that appeared at least three times among the top discriminating VOCs (compounds indicated in dark black colour, Fig. 6) were *α*-pinene, sabinene, *β*-pinene, *β*-myrcene, 2-carene, *α*-phellandrene, 3-carene, *α*-terpinene, *β*-phellandrene, (*Z*)-*β*-ocimene, (*E*)-*β*-ocimene, linalool, allo-ocimene, methyl salicylate, (*E*)-*β*-caryophyllene, and *α*-humulene (Fig. 6). Eight of the selected compounds were detected in the larval frass volatiles (Table 2, Fig. 4), which also attracted the parasitoid (Fig. 2). We therefore focused on these discriminating VOCs to test the parasitoid responses to synthetic compounds.

Table 2 Volatile organic compounds detected in *Tuta absoluta* larval frass (*n* = 5)

Peak No.[a]	RT (min)	RI[b]alk	RI[c]lit	Compound[d]	Comparison to head-space tomato plant volatiles[e]	Chemical class
1	11.08	994	992	β-myrcene*	+	Monoterpene
2	11.25	1002	1001	2-carene*	+	Monoterpene
3	11.41	1011	1011	3-carene*	+	Monoterpene
4	11.52	1017	1018	α-terpinene*	+	Monoterpene
5	11.69	1027	1026	*p*-cymene*	+	Monoterpene
6	11.80	1032	1032	β-phellandrene*	+	Monoterpene
7	14.60	1201	1197	methyl salicylate*	+	Benzenoid ester
8	14.94	1220	1218	β-cyclocitral	+	Monoterpene
9	16.68	1340	1342	δ-elemene	+	Sesquiterpene
10	17.40	1388	1385	β-elemene*	+	Sesquiterpene
11	17.80	1430	1427	(*E*)-β-caryophyllene*	+	Sesquiterpene
12	18.04	1450	1444	guaiadiene-6,9	+	Sesquiterpene
13	18.24	1466	1460	α-humulene*	+	Sesquiterpene
14	18.64	1490	1488	(*E*)-β-ionone*	+	Ketone
15	19.60	1590	1589	caryophyllene oxide	−	Sesquiterpene

[a]Peak numbers correspond to peaks indicated in Fig. 4

[b]Retention index relative to C_8–C_{30} *n*-alkanes of a HP-5MS capillary column

[c]Retention index obtained from the literature (e.g. Khan et al. 2012)

[d]Identification of compounds based on the retention time (RT), retention indices and mass spectra using NIST11, Wiley9, Adams and Chemecol, and comparison with published mass spectra and retention indices from online NIST library. * indicates compounds confirmed with authentic standards

[e]Compounds found in the volatiles of both plant and frass are indicated by a (+) while those found in the frass volatile but absent in the plant volatile are indicated by a (−)
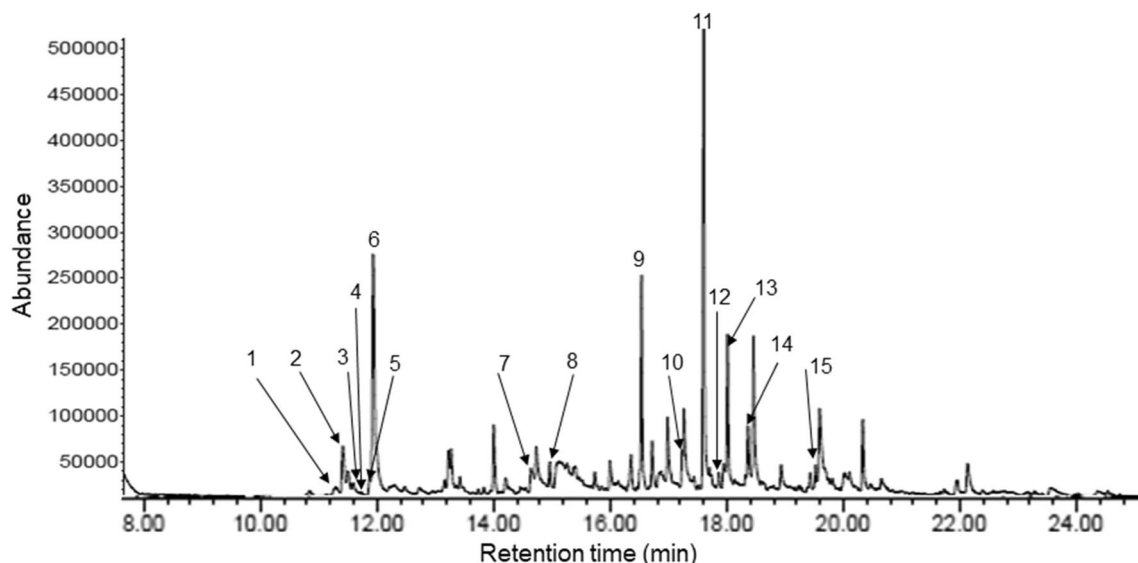

Fig. 4 GC–MS profile of *Tuta absoluta* larval frass volatiles. Numbers correspond to names of the volatile compounds listed in Table 2

Bioassays with synthetic compounds

The parasitoid *D. gelechiidivoris* was attracted to α-pinene at doses of 123 ng ($\chi^2 = 5.22$, df = 1, $P = 0.022$) and 1230 ng ($\chi^2 = 8.16$, df = 1, $P = 0.004$), but not at a dose of 12.3 ng ($\chi^2 = 0.73$, df = 1, $P = 0.391$) compared to the control (DCM) (Fig. 7). Similarly, α-phellandrene did not attract the parasitoid at a dose of 15.4 ng, while higher doses of 154 ng
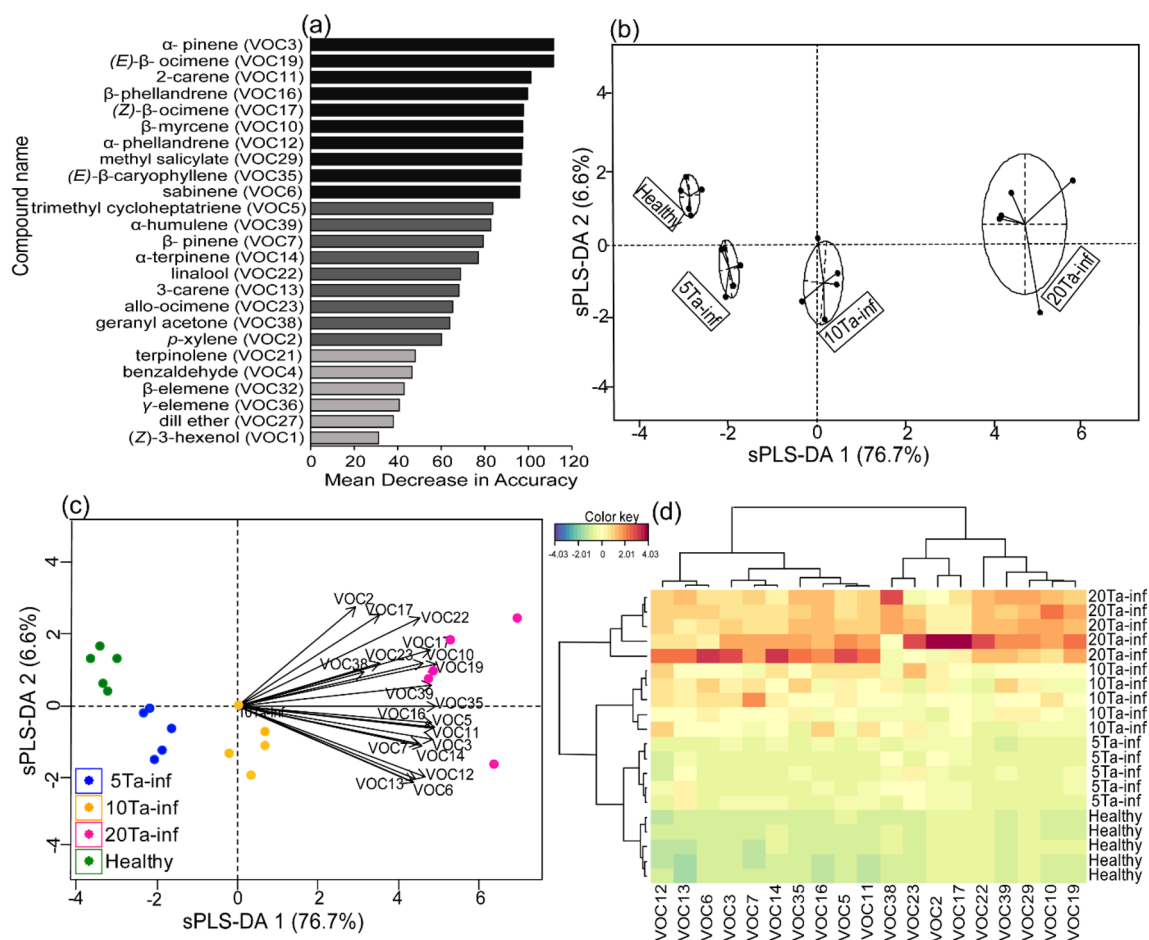
Fig. 5 Determination of the most discriminating volatiles and their correlation with healthy tomato plants and tomato plants infested with 5, 10 and 20 *Tuta absoluta* larvae (abbreviated 5Ta-inf, 10Ta-inf and 20Ta-inf, respectively, with five replicates). **a** The 25 most discriminating VOCs between healthy and infested tomato plants are listed in decreasing importance based on the mean decrease in accuracy in the random forest analysis with a classification accuracy of 100%. **b** A sparse partial least square discriminant analysis

(sPLS-DA) plot displaying the distribution of healthy and infested tomato plants (R2X = 0.789, R2Y = 0.594, Q2 = 0.532), and **c** sPLS-DA biplot showing the correlation of VOCs with healthy and infested tomato plants using the 19 top discriminating VOCs (mean decrease in accuracy, MDA ≥ 60) (R2X = 0.789, R2Y = 0.594, Q2 = 0.532). (d) Clustering heatmap showing the abundance (in decreasing colour intensity) of the top discriminating VOCs across replicates of healthy and *T. absoluta*-infested tomato plants

($x^2 = 5.78$, df = 1, $P = 0.016$) and 1.54 µg ($x^2 = 8.16$, df = 1, $P = 0.004$) attracted the parasitoid compared to the control. Attraction of *D. gelechiidivoris* to β-ocimene occurred only at a dose of 320 ng ($x^2 = 8.82$, df = 1, $P = 0.003$), but no attraction was observed for lower doses of 32 ng ($x^2 = 0.567$, df = 1, $P = 0.33$) and 3.2 ng ($x^2 = 0.5$, df = 1, $P = 0.479$) compared to the control. Likewise, β-caryophyllene at 1000 ng dose ($x^2 = 7.22$, df = 1, $P = 0.007$) attracted the parasitoid, whereas a dose of 100 ng ($x^2 = 2.52$, df = 1, $P = 0.112$) or 10 ng ($x^2 = 0.5$, df = 1, $P = 0.479$) compared to the control did not. *Dolichogenidea gelechiidivoris* was also attracted to 260 ng methyl salicylate ($x^2 = 5.78$, df = 1, $P = 0.016$), 580 ng β-myrcene ($x^2 = 6.61$, df = 1, $P = 0.01$) and 850 ng α-terpinene ($x^2 = 4.17$, df = 1, $P = 0.041$), but it was not attracted to lower doses of one-tenth and one-hundredth

compared to the control (Fig. 7). On the other hand, sabinene, β-pinene, 2-carene, 3-carene, β-phellandrene, linalool, allo-ocimene and α-humulene were not attractive to the parasitoid at doses tested in our experiments (Fig. 7).

Surprisingly, the parasitoid *D. gelechiidivoris* was not attracted to the blend of the seven attractive compounds (α-pinene, β-myrcene, α-phellandrene, α-terpinene, β-ocimene, methyl salicylate and (E)-β-caryophyllene) when mixed at their most attractive doses (blend B1) compared to the control (DCM) ($x^2 = 1.02$, df = 1, $P = 0.213$) (Fig. 8). However, subsequent dilutions of B1 elicited varying degrees of attraction in the parasitoid. The blend B2 composed of half of the B1 dose was relatively attractive to the parasitoid ($x^2 = 6.02$, df = 1, $P = 0.014$), whereas the blend B3 composed of one-fourth of the B1 dose was the most

attractive to the parasitoid ($\chi^2 = 11.76$, df $= 1$, $P = 0.0006$) compared to the control. The parasitoid was also attracted to the blend B4 composed of one-tenth of B1 dose ($\chi^2 = 9.19$, df $= 1$, $P = 0.002$), but not to blend B5, i.e. one-hundredth of B1 dose ($\chi^2 = 0.5$, df $= 1$, $P = 0.48$) when compared to the control (Fig. 8).

Discussion

We report the attractiveness of the braconid parasitoid *D. gelechiidivoris* to tomato volatiles-induced by feeding of *T. absoluta* larvae, and to the host kairomones, and identify the attractive compounds using a Y-tube olfactometer. Y-tube olfactometer and wind tunnel are both suited for the study of behavioural responses of braconid parasitoids to semiochemicals, as seen in the parasitoids *Aphidius ervi* Haliday, *Agathis bishopi* (Nixon) and *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) which were found to be active both in wind tunnel and Y-tube olfactometer, orienting more towards volatiles of host-infested plants than to those of healthy plants (Du et al. 1996; Steinberg et al. 1992; Zimba et al. 2015). In our study, we only observed the choice of odours by *D. gelechiidivoris*, and no other foraging behaviours like landing on sources, flight capacity, use of visual cues, take off ability, etc. for which the use of wind tunnel is mandatory.

Tuta absoluta larvae-infested tomato plant volatiles attracted the parasitoid *D. gelechiidivoris* because of the quantitative and qualitative differences in the volatile composition of infested tomato plants relative to healthy plants. We found that *D. gelechiidivoris* was more attracted to volatiles of tomato plants infested with *T. absoluta* larvae than to volatiles of healthy tomato plants. This is in line with findings obtained with closely related species. Suckling et al. (2012) reported that, compared to volatiles of healthy apple (*Malus domestica*) seedlings, the parasitoid *Dolichogenidea tasmanica* (Cameron) (Hymenoptera: Braconidae) was more attracted to volatiles of apple seedlings infested with the light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae). Our findings also showed that *D. gelechiidivoris* olfactory attraction was greater towards plants with high host infestation density, i.e. 20 T. *absoluta* larvae than to those with low (5 or 10) host infestation density. Such positive density-dependent olfactory responses for volatiles of host-infested plants have also been reported in other plant-host-parasitoid tritrophic interactions. For example, *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae) was reported to prefer volatiles of cabbage plants infested with a high density (15–30 larvae) of its host, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) when compared to volatiles of plants with a low (5) infestation density (Shiojiri et al. 2010; Girling et al. 2011). With no surprise, we found that *D. gelechiidivoris* was not attracted to volatiles of healthy tomato plants when compared to clean air. The same pattern has been reported in other natural enemies searching for *T. absoluta* in tomato plants, as for the parasitoid *Trichogramma achaeae* which was not attracted to volatiles of healthy tomato plants compared to clean air (Gontijo et al. 2019).

Responses of natural enemies to specific host-infested plants are a result of quantitative and qualitative differences in the compositions of plant volatiles (De Moraes et al. 1998; McCormick et al. 2012). The behavioural responses of *D. gelechiidivoris* and the tomato plant volatile emission rates were found to be positively correlated with the infestation density of *T. absoluta* larvae. We found that the density of infesting *T. absoluta* larvae led to quantitative variations in the volatile composition between healthy and infested tomato plants, as previously reported by De Backer et al. (2015). Specifically, in our study, increasing the level of *T. absoluta* larval infestation positively correlated with increased emission of volatiles, namely the monoterpenes *α*-pinene, *β*-myrcene, *α*-phellandrene, *α*-terpinene and (*E*)-*β*-ocimene, and the sesquiterpene (*E*)-*β*-caryophyllene. The increase in the rate of volatile emissions upon leaf mining by *T. absoluta* larvae could be explained by the increase in feeding intensity and the plant defence pathway activated by the insects. Indeed, leaf mining larvae are known to activate both jasmonic acid (JA) and salicylic acid (SA) pathways which may act synergistically to induce volatile emission in host plants (Yang et al. 2020). Some authors reported that activation of JA pathway in tomato plants led to the upregulation of defence genes and enhanced the production of secondary metabolites and herbivore-induced volatiles in the plants (Chen et al. 2006; Degenhardt et al. 2010). The induced JA level and the volatile emission were reported to be positively correlated with the duration and intensity of herbivore feeding, as seen in the armyworm *Spodoptera exigua* (Hübner) and *S. littoralis* (Boisd.) (Lepidoptera: Noctuidae) larvae of which feeding on maize plants resulted in the increase in both JA level and emission of terpene volatiles, as the infestation density and feeding duration increased (Schmelz et al. 2003; Turlings et al. 2004). Qualitative differences have also been observed in plant volatiles whereby herbivory on plants likely results in the emission of herbivore-specific plant volatiles (De Moraes et al. 1998; McCormick et al. 2012). We found that *T. absoluta* larvae feeding at a high density on tomato plants led to the emission of novel compounds that were not detected in the headspace volatiles of healthy tomato plants. These VOCs included the alcohol (*Z*)-3-hexenol; the ester (*Z*)-3-hexenyl butanoate; the benzenoid ester methyl salicylate; the monoterpenes sabinene, linalool and allo-ocimene; and the sesquiterpene β-elemene. Silva et al. (2017) reported that the volatile compounds specific to *T. absoluta* larvae-infested tomato plants
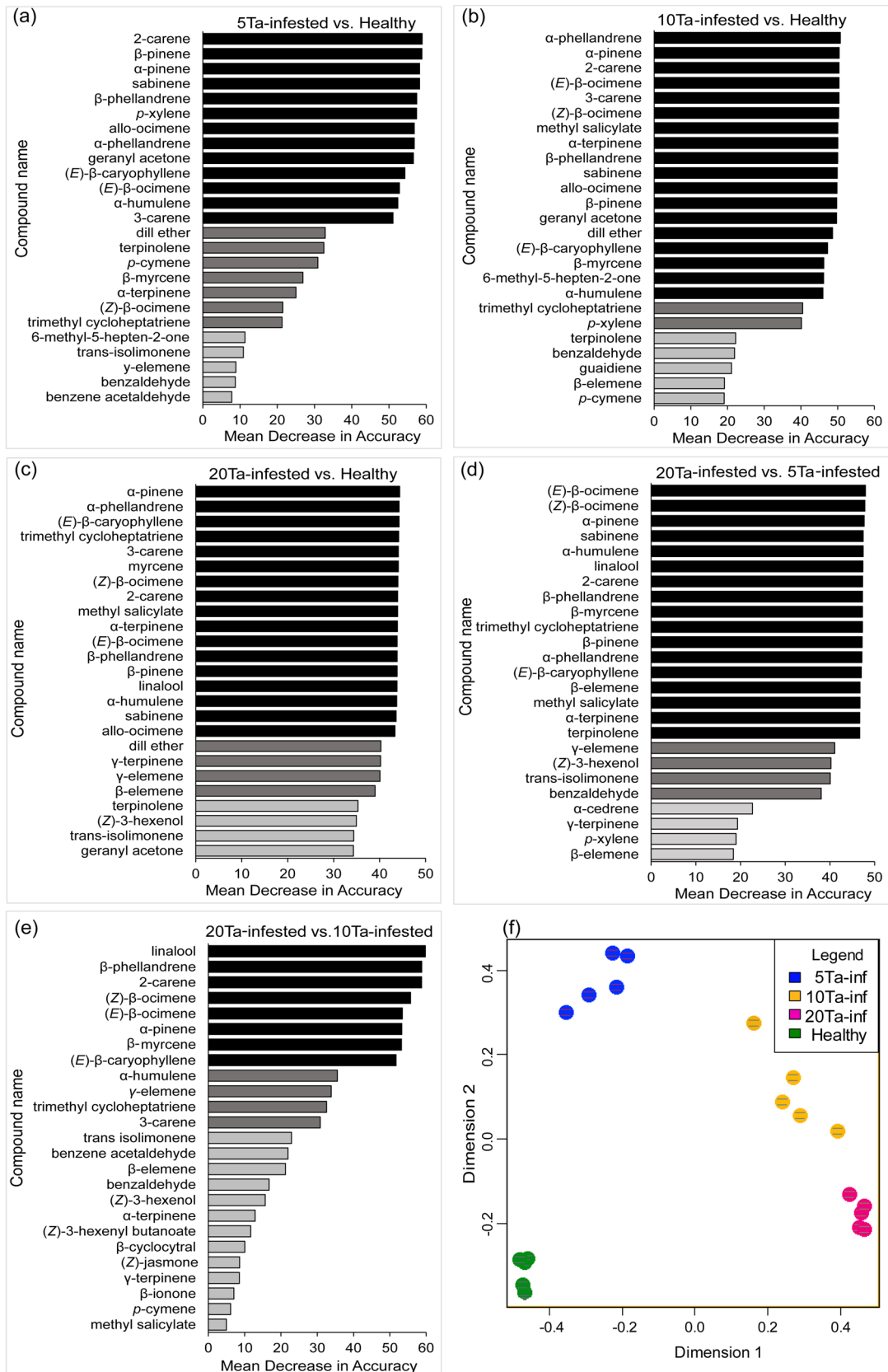
◀**Fig. 6** The 25 most predictive volatiles are listed in order of decreasing importance (difference in colour intensity) based on mean decrease in accuracy in the random forest analysis using VOC concentrations. Histogram showing volatile compounds that distinguish: **a** 5 *Tuta absoluta* (Ta) larvae-infested plants versus healthy plants; **b** 10 T. *absoluta* larvae-infested plants versus healthy plants; **c** 20 T. *absoluta* larvae-infested plant versus healthy plants; **d** 20 T. *absoluta*-infested plant versus 5 T. *absoluta*-infested plants; **e** 20 T. *absoluta* larvae-infested plants versus 10 T. *absoluta* larvae-infested plants (for each analysis, the classification accuracy was ≥95%). **f** Multidimensional Scaling (MDS) ordination plot associated with RF analysis, showing the distribution of healthy plants and plants infested with varying densities of *T. absoluta* larvae, based on the concentrations of their VOCs

were mainly alcohols: 3-methylbutan-1-ol and (*Z*)-2-penten-1-ol; and esters such as (Z)-2-penten-1-yl acetate ester, (Z)-2-penten-1-yl butyrate and (Z)-3-hexen-1-yl crotonate, but emissions of novel monoterpenes and sesquiterpenes were not observed. The differences in the specific *T. absoluta* larvae-induced tomato plants volatiles between our study and that of Silva et al. (2017) could be because of differences in the tomato cultivars used (Kilele in this study vs. Moneymaker in the study by Silva et al. (2017)), consistent with Proffit et al. (2011) who reported qualitative differences in the volatile compounds from tomato plant cultivars.

Herbivory-induced plant volatile compounds, either individually or as a blend, mediate the attraction of natural enemies to host plants (Turlings and Erb 2018; Ayelo et al. 2021). We found that the parasitoid *D. gelechiidivoris* was attracted to *α*-pinene, *β*-myrcene, *α*-phellandrene, *α*-terpinene, *β*-ocimene, (*E*)-*β*-caryophyllene, of which the emission rate increased with an increase in the intensity of *T. absoluta* herbivory, and to methyl salicylate, a volatile specifically induced by herbivory. These attractants have also been reported to act as kairomone for other natural enemy species. For example, the braconid parasitoid *A. ervi* was attracted to methyl salicylate and (*E*)-*β*-caryophyllene identified in the volatiles of tomato plants infested by *Macrosiphum euphorbiae* (Sasso et al. 2009), and to α-phellandrene and (*E*)-*β*-ocimene detected in *Acyrthosiphon pisum*-infested bean plant volatiles (Takemoto and Takabayashi 2015). Moreover, the parasitoid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) was attracted to α-pinene identified in *Heliothis virescens* (Fab.) (Lepidoptera: Noctuidae) larvae-infested cotton plant volatiles (Morawo and Fadamiro 2014). The attractive compounds were active at different equivalent plant doses. While the responses of *D. gelechiidivoris* to (*E*)-*β*-caryophyllene, α-phellandrene and α-pinene slightly increased between doses corresponding to the release rates (i.e. dose/h) of 10 and 100 equivalent plants, the other attractants were active only at release rates of 100 equivalent plants. The increase in doses to the release rates of 10 and 100 equivalent plants is guided by the expectation that in a tomato field, thousands

of plants older than those used in our bioassays continuously release volatiles to attract natural enemies. Volatile emission by tomato plants has been reported to increase with increasing plant age (Jansen et al. 2008), and with increased temperature (Copolovici et al. 2012). We thus speculate that volatile release rate in a tomato field could be higher than that of plants in the laboratory. Furthermore, as odour plume concentration is diluted by the wind over distances in the field, it is likely that kairomone doses to be applied to attract natural enemies in the field would be higher than doses that elicited attraction when tested in laboratory assays (Takemoto and Takabayashi 2015). In this regard, the increase in volatile concentrations in the dose–response assays could help get insights into the threshold concentrations detectable by, and attractive to, the parasitoid *D. gelechiidivoris* for future field application purposes. We observed that the parasitoid did not display an avoidance or repellent behaviour to the doses tested, indicating that these doses were in the relevant range for the parasitoid attraction. We found that the highest attraction for *D. gelechiidivoris* was obtained with the 7-component blend (containing 308 ng α-pinene, 145 ng *β*-myrcene, 385 ng α-phellandrene, 213 ng α-terpinene, 80 ng *β*-ocimene, 250 ng (*E*)-*β*-caryophyllene and 65 ng methyl salicylate) that elicited a similar attraction level as the volatiles of 20-*T. absoluta* larvae-infested tomato plant which was the most attractive to the parasitoid among all the infested plants. Similarly, the parasitoid *Lytopylus rufipes* Nees (Hymenoptera: Braconidae) showed the strongest attraction to a blend of five compounds, i.e. (*Z*)-3-hexenyl acetate, linalool, (*E*)-*β*-ocimene, (*E*)-3,8-dimethyl-1,4,7-nonatriene and (*E,E*)-α-farnesene (Liu et al. 2019). Insects use blend of odourants when searching for hosts in nature (Thomas-Danguin et al. 2014; Conchou et al. 2019), and odourant mixtures elicit faster olfactory processing responses in insects and are more reliable for insect olfaction than single odourants (Chan et al. 2018). However, the attractiveness of an odourant blend depends on its composition, and the concentration and ratio of the individual compounds (Beyaert et al. 2010; Cha et al. 2011). The non-attraction of the parasitoid to the blend of the seven attractants when mixed at their most attractive doses (blend B1) could be explained by the composition and the high concentration of the blend B1. High concentrations of an attractant can turn into eliciting a neutral or even repellent behaviour from natural enemies (Ren et al. 2017 and references therein). Goelen et al. (2021) reported that a blend of benzaldehyde and styrene (mixed at their most attractive doses) was attractive to the parasitoid, whereas a 5-component blend containing benzaldehyde and styrene at their most attractive doses did not attract the parasitoid at any of the doses tested. In a blend of attractants, some molecules can exert an inhibitory effect on the activity of another molecule depending on their concentrations and ratios, thereby
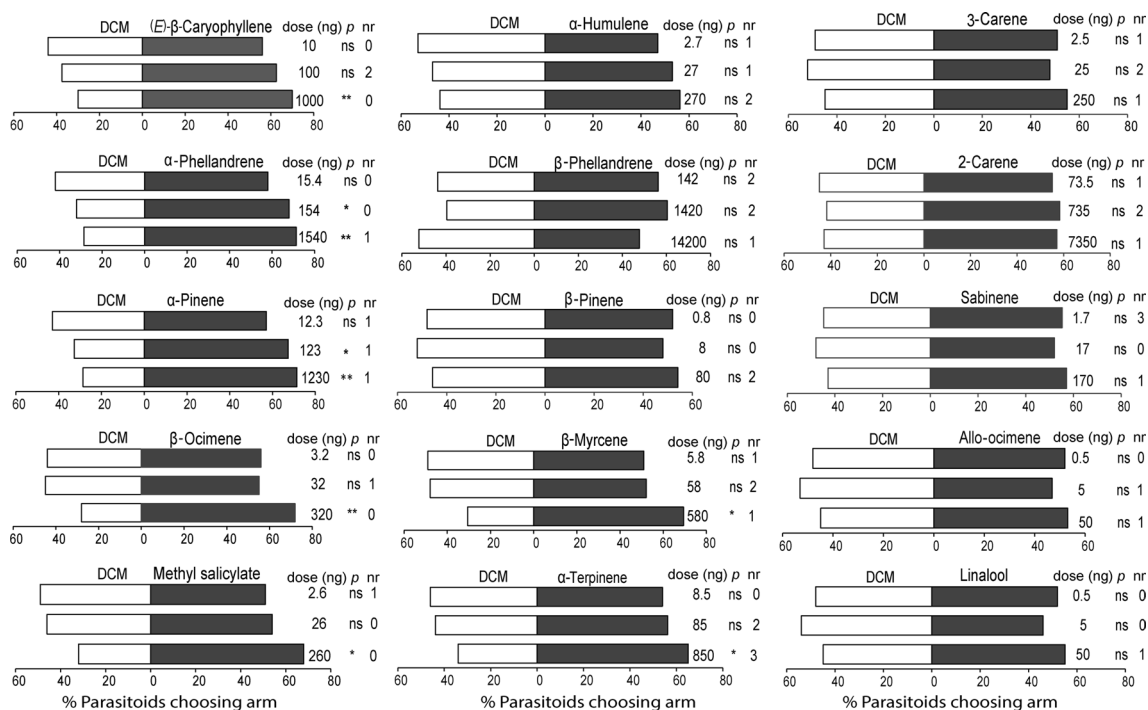
Fig. 7 Behavioural responses of *Dolichogenidea gelechiidivoris* to synthetic compounds tested at three doses corresponding to release rates by 1, 10 and 100 equivalent plants in one hour. Fifty insects were tested per choice test. n_r = number of non-responsive insects (i.e. insects that made no choice). DCM = dichloromethane. P = statistical significance level with n_s = no significant difference ($P > 0.05$); *, ** = significant differences at $P < 0.05$ and $P < 0.01$ from χ^2 test at $\alpha = 0.05$
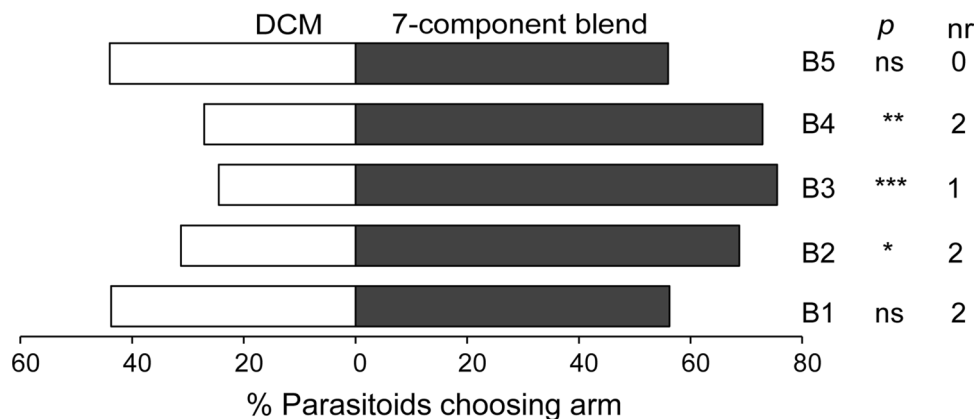
Fig. 8 Behavioural responses of *Dolichogenidea gelechiidivoris* to a 7-component blend of the attractive compounds mixed at their most attractive doses (B1) which is subsequently diluted to one-half (B2), one-fourth (B3), one-tenth (B4), and one-hundredth (B5). Fifty insects were tested per choice test. n_r = number of non-responsive insects (i.e. insects that made no choice). DCM = dichloromethane. P = statistical significance level with n_s = no significant difference ($P > 0.05$); *, **, *** = significant differences, respectively, at $P < 0.05$, $P < 0.01$ and $P < 0.001$ from χ^2 test at $\alpha = 0.05$

reducing the overall detection and the excitatory activity of the blend on the insect's olfactory neurons (Hatano et al. 2015).

Natural enemies also locate their hosts and prey using kairomones emanating from them, and known to be released from diverse sources including larval frass, glandular defensive larval secretions, and pheromones (Afsheen et al. 2008).

The detection of host-associated volatile components by *D. gelechiidivoris* may enhance the parasitoid foraging behaviour in finding *T. absoluta* on host plants in the field. Indeed, in this study, we demonstrated that volatiles of the larval frass of *T. absoluta* attracted *D. gelechiidivoris*. Similarly, Chuche et al. (2006) reported that the parasitoid *Dibrachys cavus* (Walker) (Hymenoptera: Pteromalidae)

was attracted to its host, *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) larval frass volatiles. In our study, with the exception of caryophyllene oxide which could have resulted from oxidation processes, all *T. absoluta* larval frass volatile compounds were identified in the headspace volatiles of the host plant. These findings corroborate those in previous studies reporting that volatiles from frass of leaf-chewing herbivores, like *Chrysolina herbacea* contained compounds of the digested materials of the host plant, *Mentha* spp., with oxidation of 1,8 cineole into hydroxy-1,8-cineoles, occurring in the insect's frass (Cordero et al. 2012; Pizzolante et al. 2017). Among the volatiles we identified in the *T. absoluta* larval frass, the monoterpenes *β*-myrcene and *α*-terpinene, the sesquiterpene (*E*)-*β*-caryophyllene, and the benzenoid ester methyl salicylate attracted *D. gelechiidivoris*.

Our findings also revealed that the parasitoid *D. gelechiidivoris* was attracted to the commercial sex pheromone of *T. absoluta*. The results are in agreement with those of previous studies reporting that the sex pheromone of *T. absoluta* attracted *Trichogramma* egg parasitoids (Ahmadi and Poorjavad 2018; Gontijo et al. 2019). *Tuta absoluta* sex pheromones are used in attract and kill, mass trapping and mating disruption control strategies for the management of the pest in the field (Megido et al. 2013). This control strategy could benefit the parasitoid *D. gelechiidivoris* which eavesdrops upon the pheromone, or harm the success of parasitisation if the parasitoid individuals are trapped by the pheromone-baited sticky traps used in tomato crop fields. However, greenhouse and field experiments are needed to confirm the attraction of *D. gelechidiivoris* to *T. absoluta* sex pheromone. This is particularly important because discrepancies were observed between laboratory and greenhouse or field results regarding the attraction of *Trichogramma* species to *T. absoluta* sex pheromones (Ahmadi and Poorjavad 2018). Still, positive results were found for the parasitoid *Telenomus euproctidis* Wilcox (Hymenoptera: Scelionidae) which was attracted to solvent extract of pheromone glands and synthetic sex pheromone of its host, *Orgyia postica* (Walker) (Lepidoptera: Lymantriidae) in both laboratory and field (Arakaki et al. 2011).

In summary, we report that *T. absoluta* larvae-infested tomato plants and the host larval frass release volatiles that are attractive to the braconid parasitoid *D. gelechiidivoris*. The attractive volatile compounds could be involved in the long-range kairomones exploited by the parasitoid in locating tomato plants where to find *T. absoluta* larvae for oviposition. Specifically, we show that *D. gelechiidivoris* is attracted to the terpenoids *α*-pinene, *β*-myrcene, *α*-phellandrene, *α*-terpinene, *β*-ocimene, (*E*)-*β*-caryophyllene, and to the benzenoid ester methyl salicylate, when tested individually or in a 7-component blend. Moreover, we demonstrate that *T. absoluta* sex pheromone elicits

attraction behaviour in the parasitoid and may therefore also serve as a kairomone for the parasitoid in finding *T. absoluta* on host plants in the field. The findings of our study thus elucidate the role of semiochemicals that could be exploited to recruit and retain the parasitoid *D. gelechiidivoris* in the field. In combination with other integrated pest management (IPM) methods, semiochemicals are being applied in crop fields to enhance biological control of insect pests. In this context, our findings open new avenues for development of kairomone-based lure to enhance the biological control of *T. absoluta* for the suppression of this pest within a holistic IPM programme.

Declaration

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