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Lemon Terpenes Influence Behavior of the African Citrus Triozid *Trioza erytreae* (Hemiptera: Triozidae)

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Abstract

The African citrus triozid, *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) is one of the primary vectors of the bacterium *Candidatus* Liberibacter spp. which causes citrus greening, a disease of global economic importance in citrus production. Despite its economic importance, little is known about its chemical ecology. Here, we used behavioral assays and chemical analysis to study the chemical basis of interaction between *T. erytreae* and one of its preferred host plants, *Citrus jambhiri*. In dual choice Y-tube olfactometer assays, lemon leaf odors attracted females but not males compared to plain air or solvent controls. However, in a petri dish arena assay, both sexes were arrested by lemon leaf odors. Coupled gas chromatography-mass spectrometry (GC/MS) analysis revealed quantitative differences in the odors of flushing and mature leaves, dominated by terpenes. Twenty-six terpenes were identified and quantified. In Petri dish arena assays, synthetic blends of the most abundant terpenes mimicking lemon flushing leaf odors elicited varying behavioral responses from both sexes of *T. erytreae*. A nine-component blend and a blend of the three most abundant terpenes; limonene, sabinene and β-ocimene arrested both sexes of *T. erytreae*. In contrast, a six-component blend lacking in these three components elicited an avoidance response in both sexes. Furthermore, both sexes of *T. erytreae* preferred the three-component synthetic blend to lemon crude volatile extract. These results suggest that lemon terpenes might be used in the management of *T. erytreae*.

Keywords *Citrus jambhiri* · *Trioza erytreae* · African citrus triozid · Kairomone · *Candidatus* Liberibacter spp. · Terpenes

Introduction

The African citrus triozid (ACT), *Trioza erytreae* Del Guercio, (Hemiptera: Triozidae), a member of the jumping plant-lice superfamily, Psylloidea (Burckhardt and Ouvrard 2012), is worldwide in distribution, and one of the major pests affecting global citrus production (Cocuzza et al. 2017). The sexes of *T. erytreae* are about 4 mm in length though females are slightly larger than males (Cocuzza et al. 2017), and feed on the flushes of the plant where the female also lays eggs (Aubert 1987; Catling 1973). A female psyllid lays up to 2000 eggs during 4 to 7 weeks (Grissa-Lebda and Sahraoui 2017) which hatch into nymphs (5–17 days) and after five nymphal stages (20–40 days), they become adults (van den Berg 1990). Newly emerged adults reach reproductive maturity (3–7 days) and mate 2–3 times a day (Catling 1973). Mating occurs any time of the day and is initiated by the male (van den Berg 1990). Soon after mating, females actively seek for adequate sites for oviposition of eggs on young leaves or at the tip of shoots (Annecke and Cilliers 1963; Moran and Buchan 1975). *Trioza erytreae* adults can live for up to 50 days in the field (Catling 1973). Field colonies consist of 50–86% females but a few male-less colonies have been recorded (Catling 1973) although under laboratory rearing conditions the sex ratio may be nearly equal (Begemann 1984). Damage on leaves is caused by nymphs and adults by feeding on plant sap (Cocuzza et al. 2017). In addition to feeding damage, *T. erytreae* is a vector of the bacteria *Candidatus* Liberibacter africanus

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(CLaf), which causes citrus greening disease and yield losses of 25–100% (Kilalo et al. 2009). Symptoms of bacterial infection in citrus trees include; misshapen, poorly colored, bitter-tasting, unmarketable fruits, and sometimes tree death (Mann et al. 2011).

Trioza erytreae and *Diaphorina citri* (Kuwayama) are the only known biological vectors of this disease (Alves et al. 2014). These vectors have greatly affected the citrus industries in the Americas, Asia (Cocuzza et al. 2017; Lewis-Rosenblum et al. 2015) and some parts of Africa (Samways 1990). In East Africa, they are responsible for the near collapse of the citrus industry with marginalised yields of 4–10 t/ha compared to global averages of 50–75 t/ha (Ekesi 2012; Kilalo et al. 2009). Similarly, the citrus industries in Brazil, China and some parts of North America including Florida are greatly threatened by *D. citri* and its associated pathogen *Candidatus* Liberibacter asiaticus (Alves et al. 2014; Catling 1970; Mann et al. 2013; Patt et al. 2014). Other host plants that support *T. erytreae* survival are the rutaceae plants *Clausena anisata, Vepris bilocularis, Murraya koenigii, Teclea nobilis* and *Calodendrum capense* (Aidoo et al. 2018).

Currently, management of the greening disease is largely aimed at the vector as the disease has no known cure (Belasque et al. 2010). These options include farm sanitation, intercropping (van den Berg et al. 1991) and use of the nymphal parasitoid, *Tamarixia dryi* (Catling 1970). These methods have not been effective in managing the pest situation. The use of insecticides though effective, has some drawbacks, including increased vector resistance, harmful effects on non-target species, environmental contamination, and relative unaffordability to small-scale farmers in Africa compared to other management methods (Geiger et al. 2010; Tiwari et al. 2011).

An alternative and potentially effective control method worth exploring is the use of semiochemicals that mediate various aspects of triozid biology such as host location, oviposition, mating and reproduction as demonstrated for *D. citri, Cacopsylla picta* and *C. melanoneura* (Gross and Mekonen 2005; Wenninger et al. 2009). Visual and olfactory signals are the predominant cues used by insects in host plant location (Burger et al. 2010). Therefore, the aim of this study was to understand how *T. erytreae* interacts with the volatiles of one of its preferred host plants *Citrus jambhiri*, commonly known as rough lemon. To achieve this, we used behavioral assays and chemical analysis.

Methods and Materials

Plants

Two-year-old seedlings (30 cm in height with ~50 leaves) of *Citrus jambhiri* Lush (rough lemon) were purchased from Kamiu nursery, Embu, Embu County, Kenya (0°5′1.56060″ S, 37°29′977″E). These seedlings were propagated from seeds of rough lemon fruits bought from local farmers. Dark, hardened, mature leaves formed the crowns of these seedlings. To obtain soft young flushes, seedlings were pruned of all their old leaves and allowed to reshoot for 3 weeks. For all assays, seedlings were used only once as test odor treatments and were pruned up to five times to obtain flushing leaves for insect rearing.

Insects

Colonies of adult *T. erytreae* were established at the Animal Rearing and Containment Unit at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville, Nairobi campus, from nymphs collected on farms at Mikinduru district (0°4′16.122” N, 37°50′22.548″ E), Meru County, Kenya. Emerged adults of *T. erytreae* were released onto seedlings in Perspex cages (75 × 65 × 75 cm) after 3 weeks post-pruning, following the appearance of new shoots. Plants were replaced monthly when eggs laid on the leaves had developed to adulthood. The insectary colony was rejuvenated quarterly, with nymphs collected from the field to reduce inbreeding. Insects were maintained under a rearing condition of 23 ± 2 °C, 75 ± 5% relative humidity and 12:12 h L:D photoperiod.

Olfactometric Assays to Lemon Odors

Two experiments were carried out to test responses of *T. erytreae* to plant volatiles. Experiment 1 utilized a glass Y-tube olfactometer with dynamic airflow. In Experiment 2, the assay was carried out in still air in a Perspex Petri dish arena. The Y-tube olfactometer had 10-cm long stem and 5-cm long arms with 2.5 cm internal diameter (Sigmascientific, Gainesville, FL, USA). In preliminary assays with flushing leaves of lemon, response times, i.e. the duration between start time and time choice was made, of 1-, 2-, 3- and 4-week old adult male *T. erytreae* were 2.5 ± 0.49 min, 1.77 ± 0.43 min, 2.14 ± 0.46 min and 3.78 ± 0.48 min (mean ± SE), respectively. For adult females of similar ages, the response times were 2.33 ± 0.52 min, 0.89 ± 0.21 min, 2.89 ± 0.52 min and 4.19 ± 0.43 min (mean±SE), respectively. As such, we selected 2-week-old adults of both sexes, which gave the fastest response times for use in all our assays.

Y-tube Olfactometric Assays to Lemon Odors

Responses of 2-week old adults of *T. erytreae* to odors of leaves of lemon seedlings were tested in the Y-tube olfactometer connected with Teflon tubing to the test odor sources which were headspace of flushing or mature lemon leaves or crude plant volatile extracts. The control arm was either an empty oven bag (25 cm × 380 cm, Baco & BacoFoil, Wrap

Film Systems Ltd., United Kingdom) or filter paper loaded with air-dried solvent dichloromethane (99% Analytical grade; Sigma Aldrich, St. Louis, MO, USA). Doses of the crude extracts representing 40, 80 and 160 leaf hour equivalent (LHE; 1 LHE = volatiles emitted by one lemon leaf per hour), respectively, were loaded on 2 × 2 cm strips of filter paper (Whatman No. 1) in dose-response choice assays against equal volumes (5 μl, 10 μl and 20 μl) of solvent control. Charcoal-filtered clean air was passed through each arm of the olfactometer at a flow rate of 127.5 ml/min and pulled out of the stem of the Y-tube by a vacuum system at 255 ml/min using a battery-powered, portable vacuum pump (assembled at the USDA/ARS-CMAVE, Gainesville, FL, USA).

Individual insects were released into the stem of the olfactometer and allowed 5 min to make a choice. The time spent at each arm was recorded. After every 5 insects, the odor sources were interchanged to eliminate positional bias. After every 10 replicates, a clean glass Y-tube and new test odor were used. Y-tubes were cleaned with Teepol odorless detergent and hot water, rinsed with acetone and distilled water and heated overnight in an oven at 150 °C before use. All insects were starved for at least 2 h prior to the bioassay. Thirty or forty insects of each sex were tested in each experiment depending on their availability. All experiments were conducted during the photophase 1300–1700 h given that *T. erytreae* is more active during the day than the scotophase (van den Berg and Deacon 1988).

Petri Dish Assays with Crude Lemon Volatile Extract

The Perspex Petri-dish arena (5.5 cm diameter × 1.2 cm high) was lined with filter paper (Whatman No.1) and divided into treatment, control and exploratory zones (Fig. 1), with the treatment and control zones (1 cm diameter × 2 cm length each) at opposite sides of the Petri dish. This area was enough to contain the spread of the solvent. The exploratory zone was the area in the Petri dish not demarcated as treatment or control (Fig. 1). The same doses as tested earlier in the Y-tube assay were tested (40 LHE, 80 LHE and 160 LHE) and representative volumes of solvent (dichloromethane) used were tested as controls. Before assays, the solvent was allowed to evaporate for 2 min. For each dose, 20 replicates for each sex were tested and each insect used only once. Insects that did not move into either treatment or control zones during the entire duration of the experiment were classified as non-respondents and were not included in data analysis. To minimize possible positional effects, the Petri dish was turned 180 ° and the filter paper changed after every five replicates. The Petri dishes were washed with Teepol odorless detergent and hot water and rinsed with distilled water and air-dried before use.

Behavioral responses of individual *T. erytreae* males and females were tracked by video recording using EthoVision XT version 8.0 video-tracking system (Noldus Information Technology B.V., Wageningen, The Netherlands) (Noldus et al. 2002). The arena was illuminated with a florescent tube (Osram Dulux S 11 W/21–840) to simulate daylight and the movements of individual insects released at the center of the Petri dish arena were recorded every 0.5 s with a digital camera (Panasonic, CCTV camera, model no. WV-CP 500/G). Grey scaling and center-point detection were used as with a dark contrast of 27–184 pixels and subject size was between 9 and 130 pixels with no pixel smoothing. Each insect was allowed 10 min to explore the arena. The movements of test insects were tracked, and the mean times spent in either odor source were generated using the EthoVision computer software.

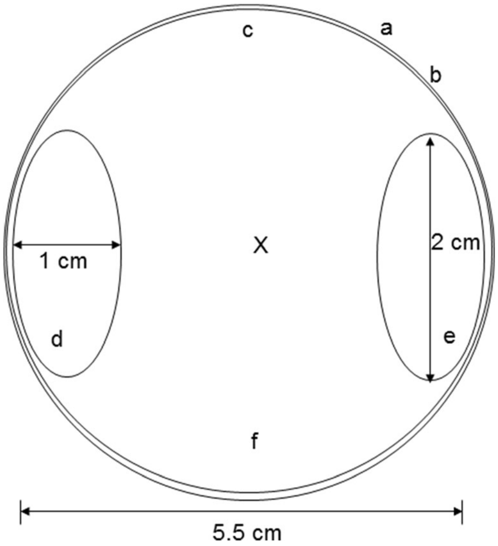

Fig. 1 Diagrammatic illustration of Petri dish arena for behavorial assay of *Trioza erytreae* to *Citrus jambhiri* crude extract and synthetic compounds. **a** Petri dish **b** Filter paper **c** Arena **d** Treatment zone **e** Control zone **f** Exploratory zone (X) Point of introduction of insect

Collection of Lemon Volatiles

Volatiles were collected in traps packed with 30 mg of Super Q adsorbent (Analytical Research Systems, Gainesville, FL, USA) by passing charcoal-purified air through lemon leaves at 350 ml/min using a push-pull system for 24 h. The entire crown of a lemon seedling was enclosed in the oven bag as described earlier. This was repeated four times on the two types of seedlings; flushing and mature. The control was an empty oven bag. Trapped volatiles were eluted using 200 μl dichloromethane (see above). The leaf sample contained ~1200 LHE. The eluate (~150 μl) was stored at −80 °C until used in bioassays.

Analysis of Lemon Volatiles

Plant volatile extracts were analyzed using coupled gas chromatography-mass spectrometry (GC/MS) on an Agilent

Technologies 7890A GC linked to a 5795C MS (inert XL/ EI/ CI MSD) triple axis mass detector, equipped with a HP5-MSI low bleed capillary column (30 m × 0.250 mm i.d, 0.25 μm) (J&W, Folsom, CA, USA). The GC oven temperature was 35 °C for 5 min with a rise of 10 °C/min to 280 °C and held at this temperature for 10.5 min. An aliquot (1 μl) of each volatile extract was injected in splitless mode, using helium as a carrier gas at a flow rate of 1.0 ml/min. Mass spectra were acquired in the electron impact (EI) ionization mode at 70 eV. The compounds were tentatively identified by comparison of their mass spectral data with library data in Adams2, Chemecol and NIST11. Synthetic standards were used to confirm the identities of compounds when available. Quantification - was based on calibration curves (peak area vs. concentration) generated from authentic standards when available and for those not available from an external standard of ethyl naphthalene.

Bioassay of Synthetic Compounds

Using differences in the identified VOCs between flushing and mature leaves (see "Results" section, Table 1), we formulated a nine-component synthetic blend out of the 22 components identified as associated with the odor of flushing leaves. These nine components were selected based on the availability of commercial standards. The nine-component blend (9CB) comprised (*S*)-(−)-limonene, sabinene, β-ocimene, myrcene, (*E*)-caryophyllene, γ-terpinene, citral (mixture of geranial and neral), terpinolene and α-phellandrene, formulated in the ratio found in the crude volatile extract ((18:4:4:2:1:1:1:1:1; Table 1). Additionally, two blends were formulated from the 9CB: a three-component blend (3CB) comprising the three most abundant compounds; (*S*)-(−)-limonene, sabinene and β-ocimene; and the remaining components combined into a six-component blend (6CB) of myrcene, (*E*)-caryophyllene, γ-terpinene, citral, terpinolene and α-phellandrene. For each blend, three doses were prepared from the optimal amounts of the individual components (Table 1) in the preferred dose (80 LHE), that is 270 ng/μl, then doubling (540 ng/μl) and quadrupling (1080 ng/μl) the amounts of the individual components and dispensed at 1 μl, 2 μl and 4 μl, respectively. The 6CB and 3CB were prepared similarly, starting with their individual amounts in the preferred dose. All the blends were tested for adult male and female *T. erytreae* responses in the Petri dish arena. The constituents of the 3CB were tested singly at their respective amounts present in the three doses.

Chemicals

α-Pinene, sabinene, myrcene, *S*-(−)-limonene, α-terpinene, *p*-cymene, γ-terpinene, terpinolene, linalool, (*E*)-caryophyllene, citral (mixture of geranial and neral), α-cedrene, α-humulene were obtained from Sigma Aldrich. A β-ocimene mixture ((*Z*)-β-ocimene, (*E*)-β-ocimene and allo-ocimene in a ratio of 2:22:1) was generously donated by the late Peter Teal (USDA/ARS-Centre for Medical, Agricultural and Veterinary Entomology, Gainesville, Florida, USA). α-Phellandrene was obtained from Bedoukian, Danbury, CT, USA. β-Bisabolene was purchased from Givaudan corporation, Clifton, NJ, USA. All the chemicals were at 95–99% purity.

Data Analyses

The time spent (min) was converted into proportions to address dependence of visiting time by *T. erytrae* to the olfactometer and Petri dish odor fields and then a log-ratio transformation ($\log_{10}$) was applied to account for compositional nature of the proportions (Aitchison 1986; Tamiru et al. 2011). The transformed data were then subjected to *t-test* to evaluate the difference between treatment and control. The analyses were implemented in R statistical software, version 3.5.1 (R Development Core Team 2015).

Results

Y-tube Olfactometric Assays to Lemon Odors

Behavioral responses of adult males to odors from both flushing and mature lemon were not significantly different (Fig. 2). By contrast, adult females were significantly attracted to both flushing ($t = 3.04$, $df = 60$, $P < 0.01$) and mature lemon ($t = 5.36$, $df = 86$, $P < 0.001$) odors compared to the controls (Fig. 2). When presented with both odor sources simultaneously, neither sex showed any preference for either mature or flushing leaf odors (Fig. 2).

Tests with the lemon leaf volatiles confirmed responses of both males and females. Males did not show significant preference for any of the doses tested (Fig. 3a). On the other hand, females were significantly attracted to the volatile extract at 80 LHE ($t = 3.29$, $df = 58$, $P < 0.01$) but not to 40 LHE ($t = 0.67$, $df = 58$, $P > 0.05$) and 160 LHE ($t = 0.84$, $df = 58$, $P > 0.05$) when compared to solvent controls (Fig. 3a).

Petri Dish Assays with Crude Lemon Volatile Extract

Of the three doses tested, males avoided 40 LHE ($t = 2.47$, $df = 38$, $P < 0.05$). However, 80 LHE elicited significant arrestment ($t = 4.47$, $df = 38$, $P < 0.001$) but not 160 LHE ($t = 1.43$, $df = 38$, $P > 0.05$) (Fig. 3b). Conversely, 40 LHE ($t = 18.96$, $df = 36$, $P < 0.001$) and 80 LHE ($t = 3.19$, $df = 34$, $P < 0.01$) elicited arrestment in females but not 160 LHE ($t = 1.90$, $df = 34$, $P > 0.05$) (Fig. 3b).

Table 1 Volatile organic compounds identified in the headspace of whole seedlings of *Citrus jambhiri* with approximately 50 flushing or mature leaves collected for 24 h from four plants

					Amount (ng/µl ± SE)		
Peak No.[1]	Compound[2]	RT (min)	RI $^{\# 3}$	RI_L ^{4}	Flush	Mature	Ratio Flush/ Mature
1	α-thujene*	9.14	906	904^e	6.74 ± 0.29	5.28 ± 0.07	1
2	α-pinene*^	9.25	912	913^e	6.95 ± 0.22	5.57 ± 0.17	1
3	sabinene*^	10.14	954	952^e	34.29 ± 1.07	12.42 ± 1.81	3
4	myrcene*^	10.54	973	974^a	12.35 ± 1.25	5.84 ± 0.14	2
5	α-phellandrene*^	10.85	988	985^a	7.43 ± 0.51	–	–
6	α-terpinene*^	11.09	999	996^a	5.37 ± 0.05	5.27 ± 0.08	1
7	*p*-cymene*^	11.17	1003	1000^a	–	5.15 ± 0.01	–
8	limonene*^	11.24	1007	1007^e	147.82 ± 20.65	13.85 ± 4.41	11
9	(*Z*)-β-ocimene*^	11.44	1018	1017^e	10.15 ± 0.57	6.15 ± 0.33	2
10	(*E*)-β-ocimene*^	11.62	1028	1027^e	31.30 ± 3.37	10.12 ± 4.58	3
11	γ-terpinene*^	11.81	1039	1041^a	10.96 ± 1.07	5.39 ± 0.12	2
12	terpinolene*^	12.39	1072	1073^a	7.66 ± 0.65	–	–
13	linalool*^	12.52	1079	1079^e	–	5.84 ± 0.38	–
14	Unidentified*	12.58	1082		6.86 ± 0.95	–	–
15	allo-ocimene*^	13.07	1111	1112^a	7.06 ± 0.40	–	–
16	citronellal*	13.41	1132	1134^c	5.68 ± 0.01	5.86 ± 0.37	1
17	geranial*^	15.24	1252	1247^c	7.71 ± 0.47	–	–
18	δ-elemene*	16.15	1315	1313^a	7.71 ± 0.62	5.45 ± 0.11	1
19	1-tetradecene*	16.87	1368	1389^d	5.36 ± 0.22	–	–
20	β-elemene*	16.96	1375	1366^a	5.62 ± 0.24	–	–
21	α-cedrene*^	17.21	1394	1391^a	–	5.35 ± 0.03	–
22	(*E*)-caryophyllene*^	17.29	1399	1396^a	11.40 ± 1.37	6.27 ± 0.18	2
23	(*Z*)-α-bergamotene*	17.44	1411	1410^c	9.84 ± 0.91	5.59 ± 0.12	2
24	α-humulene*^	17.74	1435	1430^a	5.78 ± 0.16	5.10 ± 0.03	1
25	bicyclogermacrene*	18.27	1476	1490^c	–	5.16 ± 0.08	–
26	(*E, E*)-α-farnesene*	18.31	1480	1496^c	–	6.12 ± 0.06	–
27	β-bisabolene*^	18.43	1489	1489^f	7.56 ± 0.52	–	–

[1] Peak numbers correspond to the peaks in Fig. 4

[2] *^Identification based on comparison of retention times (RT) with those of commercially purchased synthetic standards, *Tentative identification by comparison of mass spectra with published mass spectral library data only

[3] RI$^{\#}$ - Retention index relative to C8-C31 n- alkanes of a HP-5 MS column

[4] RI_L - Retention index obtained from literature: [a] Njuguna et al. 2018; [b] Kartal et al. 2007; [c] Babushok et al. 2011; [d] Mimica-Dukić et al. 2003; [e] Pavlović et al. 2006; [f] Gkinis et al. 2003

Analysis of Volatiles

A total of 27 compounds were detected in the leaf volatile profiles (Fig. 4, Table 1), and of these, the identities of 18 components were confirmed using authentic standards. The compounds identified were predominantly terpenes and the amounts varied quantitatively between flushing and mature leaves (Table 1). The three monoterpenes limonene, sabinene and β-ocimene stood out as the most abundant compounds representing almost 64% and 34% of the volatiles emitted by flushing and mature leaves, respectively, and they were 11-, 3- and 3-fold more abundant in flushing than in mature leaf odors (Table 1).

Responses to Synthetic Compounds in Petri-Dish Arena Assays

Both sexes of *T. erytreae* were significantly arrested by all the three doses of the 9CB: at 270 ng/µl, male ($t = 3.19$, $df = 38$, $P < 0.01$), female ($t = 4.99$, $df = 36$, $P < 0.001$); at 540 ng/µl, male ($t = 5.08$, $df = 38$, $P < 0.001$), female ($t = 7.01$, $df = 36$, $P < 0.001$); and at 1080 ng/µl, male ($t = 4.12$, $df = 38$, $P < 0.001$) and female ($t = 4.03$, $df = 36$, $P < 0.001$) (Fig. 5a). In contrast, all the tested doses of the 6CB were significantly avoided by both sexes of *T. erytreae* (Fig. 5b). At 57 ng/µl, male ($t = 2.58$, $df = 38$, $P < 0.05$), female ($t = 6.41$, $df = 38$, $P < 0.001$); at 114 ng/µl, male ($t = 5.25$, $df = 38$, $P < 0.001$),

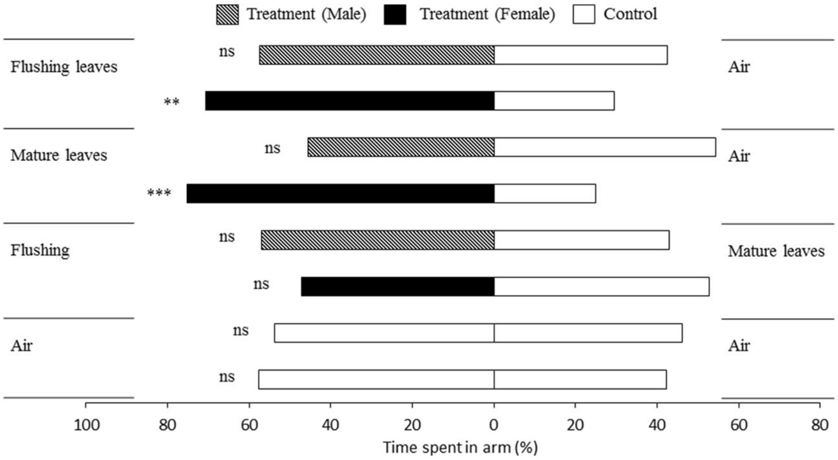

Fig. 2 The percentage time spent by male and female *Trioza* erytreae ($N = 30–40$) in the treatment arm with odors from whole *Citrus jambhiri* seedlings with flushing and mature leaves against clean air controls in a Y-tube olfactometer. The difference between time spent in treatment and control was evaluated using *t-test*. *** indicates significance at $P < 0.001$, ** indicates significance at $P < 0.01$, ns indicates not significant at $P > 0.05$

female ($t = 5.76$, $df = 36$, $P < 0.001$); and at 228 ng/µl, male ($t = 4.55$, $df = 38$, $P < 0.001$) and female ($t = 13.03$, $df = 36$, $P < 0.001$) (Fig. 5b).

For the 3CB, males were significantly arrested by 213 ng/µl ($t = 7.98$, $df = 38$, $P < 0.001$) and at 426 ng/µl both sexes were arrested; male ($t = 6.14$, $df = 58$, $P < 0.001$) and female ($t = 5.44$, $df = 38$, $P < 0.001$) (Fig. 5c). The responses of both sexes to the highest dose, 852 ng/µl, and the control were not significantly different.

Both sexes responded differently to varying doses of the three individual components. Males were significantly arrested by (*S*)-(−)-limonene at 148 ng/µl ($t = 7.21$, $df = 38$, $P < 0.001$) but avoided the dose at 592 ng/µl ($t = 3.15$, $df = 38$, $P < 0.01$) (Fig. 6a). However, females did not show any significant preference for limonene tested at the three different doses (Fig. 6a). In contrast, both sexes were indifferent to the three tested doses of sabinene (Fig. 6b). For *β*-ocimene, whereas females avoided the dose tested at 62 ng/µl, ($t = 2.57$, $df = 36$, $P < 0.05$) males responded similarly at double this dose, 124 ng/µl ($t = 2.26$, $df = 36$, $P < 0.05$) (Fig. 6c).

In the assays, both sexes of *T. erytreae* were significantly arrested by the 3CB when tested against the most preferred dose 80 LHE of the crude extract; male ($t = 3.97$, $df = 32$, $P < 0.001$) and female ($t = 5.26$, $df = 38$, $P < 0.001$) (Fig. 7).

Discussion

Our results clearly show that both sexes responded to lemon leaf odors, albeit at different levels. However, females were more responsive than males in the Y-tube olfactometer assays to odors of the living plants, whereas in the Petri-dish assays with leaf volatile extracts, both sexes responded similarly to the odors. These results suggest that females may be more sensitive to detecting lemon leaf odors than males. However, at close range, as demonstrated in the Petri-dish assays, both sexes detected lemon leaf odors. Moran and Brown (1973) reported that there was no sexual dimorphism in the antenna of *T. erytreae* as they bore the same type and number of sensoria, but several other reasons may account for this

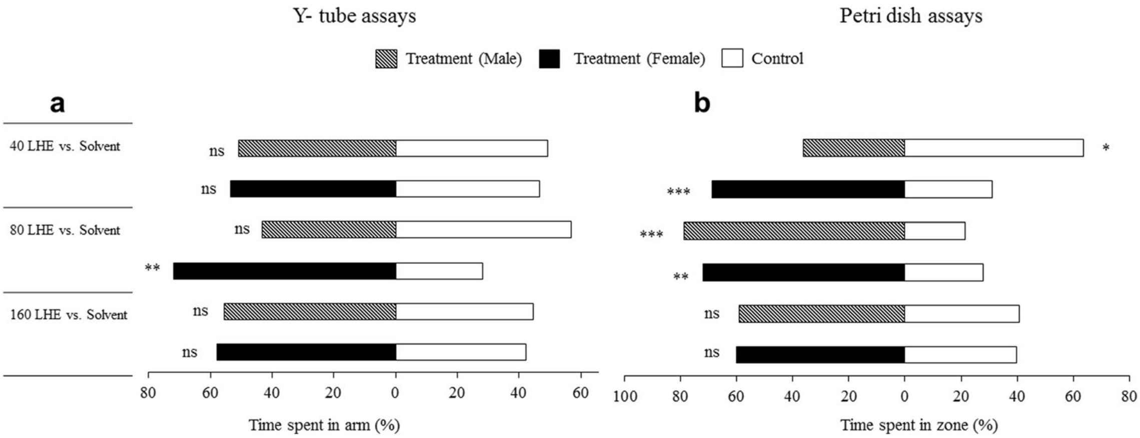

Fig. 3 The percentage time spent by 2-week-old male and female *Trioza erytreae* ($N = 20–30$) in the treatment arm/ zone (40 LHE, 80 LHE and160 LHE dose of the flushes of *Citrus jambhiri* eluate) versus control solvent of the Y-tube olfactometer (**a**) and Petri dish (**b**), where LHE is Leaf Hour Equivalent. The difference between time spent in treatment and control was evaluated using *t-test*. *** indicates significance at $P < 0.001$, ** indicates significance at $P < 0.01$, * indicates significance at $P < 0.05$, ns indicates not significant at $P > 0.05$

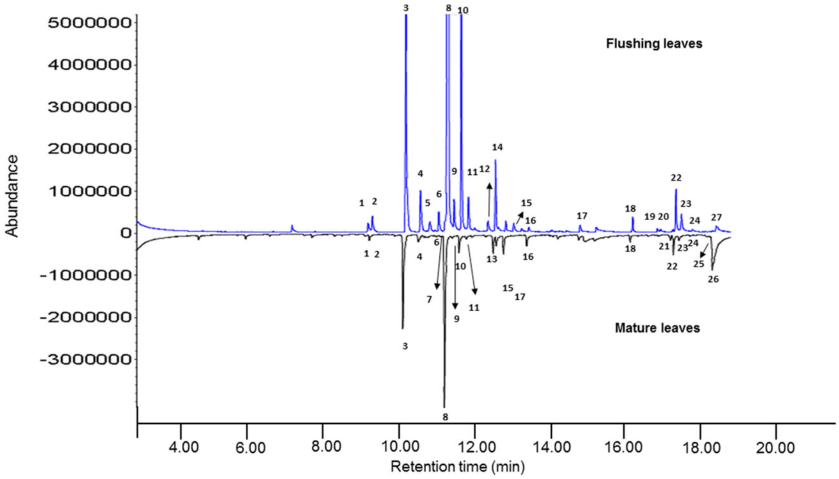

Fig. 4 GC/MS profiles of leaf volatile extracts from flushing and mature leaves. Numbers correspond to compounds listed in Table 1

differential response between the sexes. Firstly, it may be associated with the need of the female to locate host plants for nutrition and a site for oviposition to sustain its progeny. van den Berg and Deacon (1988) reported that females have a higher propensity than males for long flights, probably due to an inherent need to find plants with new shoots on which to oviposit whereas males exert energy in finding females for mating (van den Berg 1990). Female responsiveness to host plant odors has been reported for other members in the superfamily psylloidae; *D. citri* (Wenninger et al. 2009), *C. melanoneura* (Gross and Mekonen 2005) and *Cacopsylla bidens* (Soroker et al. 2004). Since mating usually occurs 3–7 days after adult emergence (Catling 1973), this suggests that adult insects used in the present study were mostly mated, which might influence their responses to test odors. In our study, we found it challenging to rear virgin individuals for the behavioral experiments because of the low survival of nymphs that were isolated singly. As such, for future studies, it would be interesting to compare responses of gravid and non-gravid females and unmated and mated males to lemon leaf odors. Secondly, it is also possible that the olfactometer conditions used in the experiments may have favored one of the sexes and not the other. Hence, there is the need for further research such as altering the airflow in the olfactometer to confirm the sex differential responses to lemon leaf odors. Nonetheless, the results of our assays confirmed our

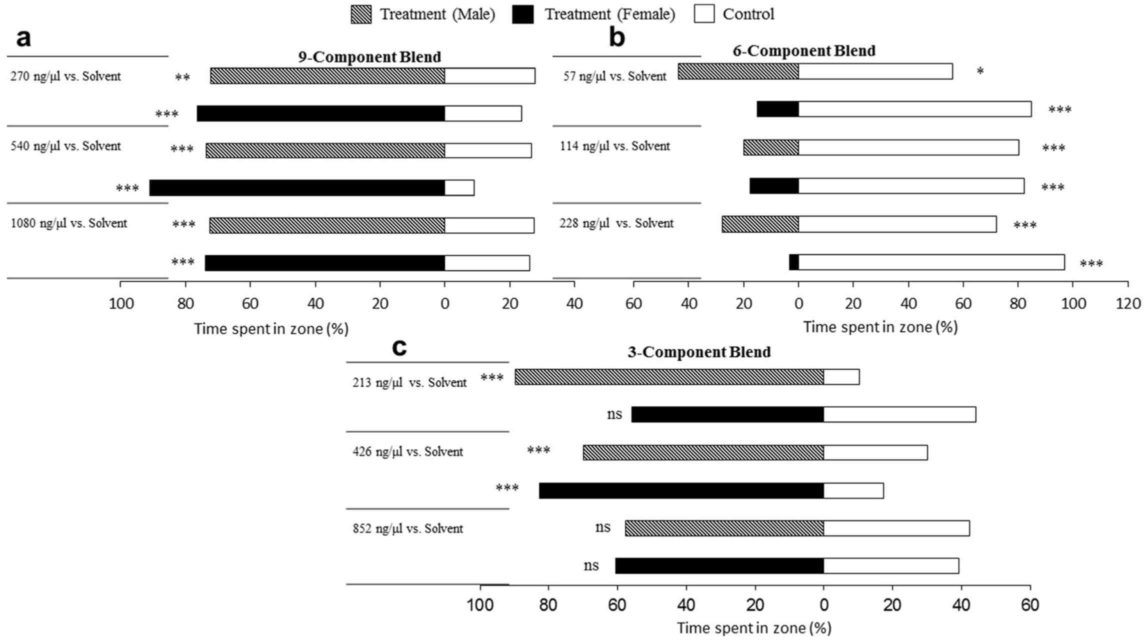

Fig. 5 The percentage time spent by 2-week-old male and female *Trioza erytreae* ($N = 20$) in the treatment zone for **a** 9-component blend, **b** 6-component blend, and **c** 3-component blend of synthetic chemicals versus control (solvent) in the petri dish bioassay. The difference between time spent in treatment and control was evaluated using *t-test*. *** indicates significance at $P < 0.001$, ** indicates significance at $P < 0.01$, * indicates significance at $P < 0.05$, ns indicates not significant at $P > 0.05$

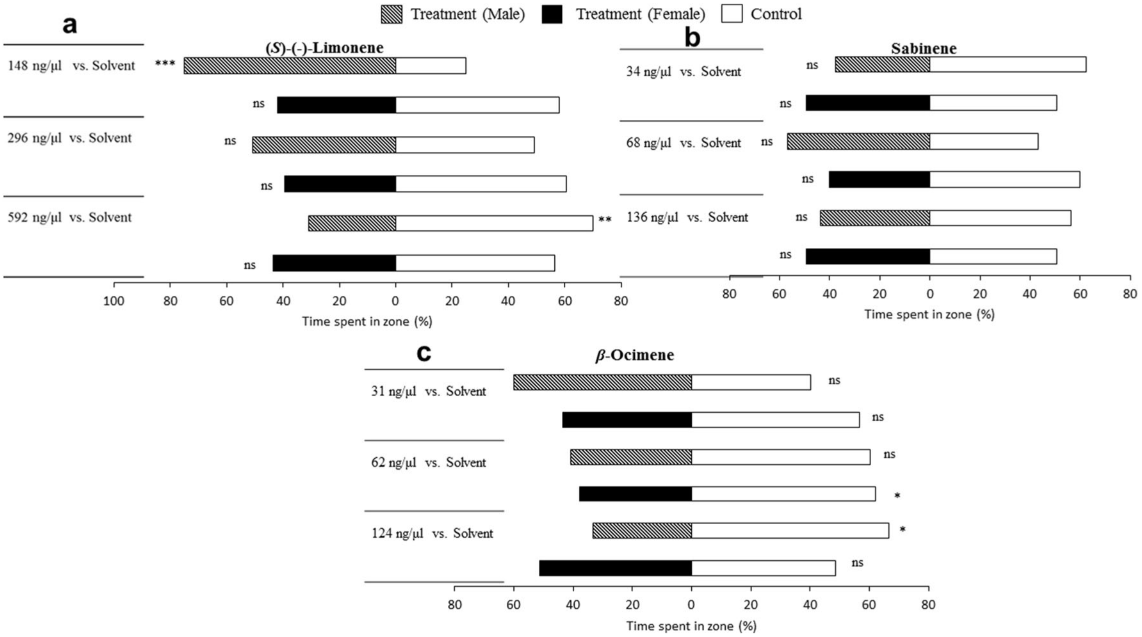

Fig. 6 The percentage time spent by 2-week-old male and female *Trioza erytreae* ($N = 20$) in the treatment zone versus control (solvent) in the petri dish bioassay of individual synthetic compounds: **a** (*S*)-(−)-limonene, **b** sabinene, **c** β-ocimene. *** indicates significance at $P < 0.001$, ** indicates significance at $P < 0.01$, * indicates significance at $P < 0.05$, ns indicates not significant at $P > 0.05$

hypothesis, which were also in agreement with previous findings by Moran and Brown (1973) that olfaction plays a role in host location in *T. erytreae.* In contrast to the present study, Moran and Brown (1973) used excised leaves of *C. limon* in a Perspex chamber and the volatiles involved in host location were not identified. This suggests that perhaps, irrespective of the lemon plant state, its headspace possesses the right balance of sensory inputs that stimulates the insect's olfactory responses (Miller and Strickler 1984; Renwick and Radke 1987). However, it would be interesting to compare the composition of the volatiles of intact and excised leaves to further understand this phenomenon.

In Y-tube olfactometer assays, both mature and flushing leaf volatiles were attractive to females, suggesting that both leaf types possess common cues that are attractive to *T. erytreae*. This concurs with earlier reports that in the absence of young flushes, which is the most preferred leaf stage for feeding and oviposition (Green and Catling 1971), mature *T. erytreae* could possibly feed on host plants but would not be able to oviposit (Cook et al. 2014; van den Berg 1990). The citrus canopy comprises both mature and flushing leaves with the latter mostly seasonal (Catling 1969). These leaf types may contribute to the attraction of *T. erytreae* to the host plant. However, after locating the host plant, the insect may decide which part to inhabit based on its needs and/or mode of feeding (Catling 1970). This choice of specific plant part may explain the availability and infestation of some insects only when those parts are present on the plant. For instance, the

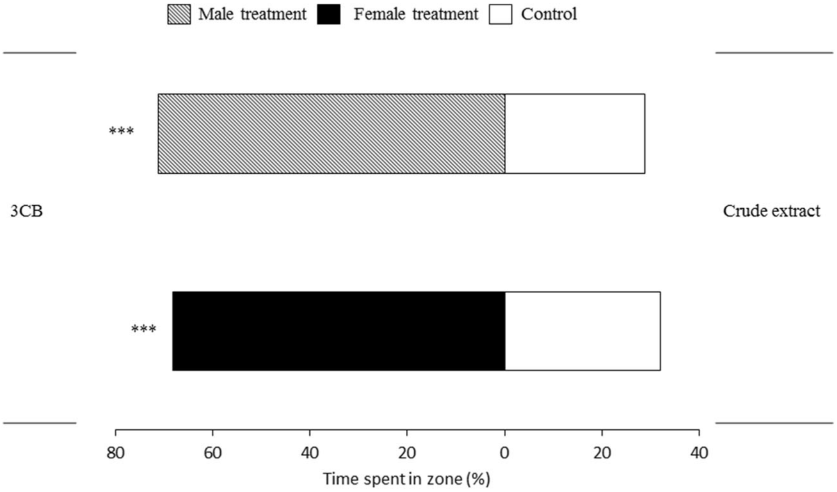

Fig. 7 The percentage time spent by 2-week-old male and female *Trioza erytreae* ($N = 20$) in the treatment zone with the preferred dose of three-component blend versus 80 LHE of crude volatile extract in the petri dish bioassay. *** indicates significance at $P < 0.001$

population peaks of *T. erytreae* are known to coincide with seasons with cool temperatures and at high altitudes where young flushes are abundant and the longevity of these flushes can be sustained (Catling 1969; Cook et al. 2014). Moran and Buchan (1975) observed that young leaves not only provided more nutrients to the pest, but were also more succulent, softer, and easier to penetrate with the stylet in comparison to old and mature leaves. The choice of host plants such as rough lemon by *T. erytreae* and *Murraya paniculata* by *D. citri*, has been reported to be influenced by the ability of the plant to produce adequate flushes (Aubert et al. 1988; Walter et al. 2012). Thus, although *T. erytreae* utilises both mature and flushing leaves, the latter are more crucial for their survival. In the present study, males could not perceive the treatment odor at the lowest dose of 40 LHE. These findings suggest that interactions between insects and their host plants are complex, and that insects may associate local volatile emission compositional differences to determine their arrestment/short- and long-range responses and other plant traits necessary for their survival. Therefore, management interventions targeting flushing regimes, which coincides with peak populations of *T. erytreae* would be a timely approach to better manage the pest.

Chemical analyses identified terpenes as the dominant compounds in the headspace of citrus, with significant quantitative differences observed between the volatile profiles of the two leaf types. Similar to our findings, Azam et al. (2013) reported that citrus leaf volatiles changed during leaf development as young leaves produce higher amounts of volatiles than mature leaves. These volatiles and their concentrations mediate various plant-herbivore interactions in several plants (Khater 2012). In the present study, the monoterpenes limonene, sabinene and β-ocimene were identified as the most abundant volatiles in the headspace of both flushing and mature leaves. It is possible that the differential responses of both sexes to flushing and mature leaf odors may be due to the increased emissions of the three compounds in a specific ratio in flushing than in mature leaf odors. These three major compounds are known components of volatiles of other citrus varieties (Azam et al. 2013; Robbins et al. 2012). The less abundant background odors also contribute to the overall attractiveness of these major volatiles as observed in the responses of *T. erytreae* to the nine-component blend. Additionally, in our study, we identified *p*-cymene, γ-terpinene, allo-ocimene, α-cedrene, α-humulene, bicyclogermacrene, and β-bisabolene, which have not been previously reported in the headspace of *C. jambhiri* (Azam et al. 2013; Robbins et al. 2012; Yamasaki et al. 2007). This finding supports the observation of variation in volatile composition between plant cultivar and plant developmental stage and part (Figueiredo et al. 2008; Njuguna et al. 2018).

In tests with the synthetic blends, the sexes responded differently. The fact that, at all the doses tested, the nine-component blend arrested both sexes to varying degrees suggests that the various terpenes at different levels may combine synergistically or additively in specific ratios to elicit a behavioral response from *T. erytreae*. This confirms previous observations that specific compounds and/or blends of volatile compounds in specific ratios play a vital role in host plant location by insects (Bruce and Pickett 2011; Tamiru et al. 2011). Interestingly, removal of the three most abundant monoterpenes; limonene, sabinene and β-ocimene from the nine-component blend to give a six-component blend, significantly altered responses of both sexes. This six-component blend elicited an avoidance response in both sexes at all the doses tested. This suggests that the three monoterpenes limonene, sabinene and β-ocimene are key signature chemical cues for host location by this triozid. Tests with the three components combined into a blend confirmed the important role they play in host location by this triozid, eliciting an arrestment response from both sexes at different doses. However, when these three components were tested singly, depending upon the chemical and dose, they elicited either an arrestment, indifferent or avoidance response from *T. erytreae.* These results suggest that for each component in lemon volatiles, a specific concentration and combination is needed to elicit a behavioral response from the different sexes of *T. erytreae* (Dudareva et al. 2003; Matias et al. 2016; Pino et al. 1999). Patt and Setamou (2010) reported that the behavioral responses of insects can be influenced by the concentration of odor, and that lower concentrations elicit attraction whereas higher ones induce aversive behavior as found in the present study with male triozids responding to (*S*)-(−)-limonene. Similar findings have been reported in other insects. For example, females of the fruit flies *Heteropsylla cubana, Drosophila melanogaster* and *D. suzukii* are sensitive to doses of their host odor and prefer lower concentrations to higher ones (Kleiber et al. 2014; Lapis and Borden 1993; Stensmyr et al. 2003). On the contrary, based on the sensilla present on the antennae, it was reported that the carrot pysllid, *Trioza apicalis* uses olfactory cues to locate the strong volatile cues emitted by their host plants (Kristoffersen et al. 2006).

We found that the blend of monoterpenes including limonene, sabinene and β-ocimene in the, same ratio at source as found in the volatiles collected from lemon leaves, arrested *T. erytreae*. Other studies have found similar results. For instance, a VOC blend which included limonene and (*E*)-β-ocimene in the citrus headspace was found to be attractive to *D. citri* (Patt and Setamou 2010). Also, (*S*)-(−)-limonene and (*E*)-β-ocimene combined with five other compounds (benzaldehyde, (*R,S*)-(±)-linalool, (*E*)-myroxide, phenylacetaldehyde, and (*R*)-(−)-piperitone) were found to attract the cotton bollworm, *Helicoverpa armigera* in wind tunnel experiments (Bruce and Cork 2001).

In *T. erytreae* both nymphs and adults live on the plant, and hence they might not require strong chemical cues for host location, but rather local quantitative differences to distinguish between flushing and mature leaves. Although, in the natural situation, *T. erytreae* can disperse up to 1.5 Km (van den Berg and Deacon 1988), wind has been implicated to play important role in its passive dispersion (Cocuzza et al. 2017; Tamesse and Messi 2004) probably due to its small size. However, arrestment cues may play important physiological roles in insects. Landolt and Phillips (1997) and Reddy and Guerrero (2004) reported that kairomones often synergize insect responses to sex pheromones.

We identified limitations in our study. First, given that *R*-(+) limonene has been associated with orange and *S*-(−)-limonene with lemon, we chose the latter for our experiments (Friedman and Miller 1971). Given the importance of chirality in determining responses of insects to semiochemicals (Singh et al. 2014; Nguyen et al. 2006), the enantiomeric composition of the limonene in our collections should be determined and the response of *T. erytreae* to the other enantiomer tested. Secondly, the compositions of the blends of synthetic chemicals applied to the filter paper in our bioassays were based on the relative amounts found in the collections of volatiles. The release rates of the volatiles loaded onto the filter paper should be measured in order to make possible testing of a blend that more closely simulates that released by the leaves of the plant.

In summary, the present study shows that olfaction mediates host location of both male and female adult *T. erytreae*. However, it appears that for both sexes, olfaction may be effective only as an arrestment signal to a blend of limonene, sabinene and β-ocimene and an avoidance signal to blends of lemon odors lacking in these three compounds. The pest management potential using these arrestment and avoidance signals needs to be evaluated in the field in combination with long range cues e.g. visual cues, for their possible integration in *T. erytreae* population suppression in citrus plantations.

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