

Behavioral Response of The Egg Parasitoid *Cosmocomoidea Annulicornis* (Hymenoptera: Mymaridae) and Characterization of The Chemical Cues That Guide Females Toward Host Eggs Located In Taxonomically Distant Plants Species

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Abstract

Parasitoids are known to exploit volatile cues emitted by plants after herbivore attack to locate their hosts. Feeding and oviposition of a polyphagous herbivore can induce emission of odor blends that differ among distant plant species, and parasitoids have evolved an incredible ability to discriminate them and locate their hosts relying on olfactive cues. We evaluated the host searching behavior of the egg parasitoid *Cosmocomoidea annulicornis* (Ogloblin) (Hymenoptera: Mymaridae) in response to odors emitted by two taxonomically distant host plants, citrus and johnsongrass, after infestation by the sharpshooter *Tapajosa rubromarginata* (Signoret) (Hemiptera: Cicadellidae), vector of Citrus Variegated Chlorosis. Olfactory response of parasitoid females toward plants with no herbivore damage and plants with feeding damage, oviposition damage and parasitized eggs was tested in a Y-tube olfactometer. In addition, volatiles released by the two host plant species constitutively and under herbivore attack were characterized. Females of *C. annulicornis* were able to detect and significantly preferred plants with host eggs, irrespectively of plant species. However, wasps were unable to discriminate between plants with healthy eggs and those with eggs previously parasitized by conspecifics. Analysis of plant volatiles induced after sharpshooter attack showed only two common volatiles between the two plant species, indole and β -caryophyllene. Our results suggest that this parasitoid wasp uses common chemical cues released by different plants after herbivory at long range and that, once on the plant, other more specific chemical cues could trigger the final decision to oviposit.

Introduction

Herbivore feeding induces the emission of volatile blends that can be exploited by natural enemies to locate their preferred hosts (Turlings et al. 1995; Dicke 1999; Clavijo McCormick et al. 2012). Hymenopteran parasitoids locate their hosts at medium to long-range by using chemical volatile cues (Vet and Dicke 1992). Consequently, parasitoids have evolved an incredible ability to discriminate among different blends of volatile organic compounds (VOCs) induced in plants by their herbivore hosts. These compounds, known as synomones, are usually emitted in larger quantities and over larger distances than semiochemicals provided by the host itself, known as kairomones. These last ones are reliable indicators of host presence, but their detectability is usually low because of the small biomass of the host in relation to that of the plant that holds them, and because of selection on the herbivores to minimize volatile emission (Fatouros et al. 2008; Clavijo McCormick et al. 2012). Apart from synomones, some parasitoids use other host-habitat cues (arrestants) or background odors produced by the herbivores (faeces, salivary secretions, silk or honeydew) or by other organisms associated with the host (Greany et al. 1977; De Moraes et al. 2000; Stuhl et al. 2011). In addition to the olfactory stimuli, visual and vibrational cues can also have an important role during the host location process (Fischer et al. 2001; Giunti et al. 2015).

Tapajosa rubromarginata (Signoret) (Hemiptera: Cicadellidae: Proconiini) is a polyphagous sharpshooter with a Neotropical distribution (Dellapé and Paradell 2013) that has been reported feeding and ovipositing on over 35 plant species in 17 families, some of commercial importance, such as *Saccharum officinarum* L., *Avena sativa* L., *Allium sativum* L., *Vitis vinifera* L., *Citrus sinensis* L., *C. limon* (L.), *C.*

unshiu and *Zea mays* L., but also on numerous weeds like *Sorghum halepense* L. (Paradell et al. 2012; Dellapé 2013; Dellapé et al. 2013; Virla et al. 2019, 2020). This sharpshooter transmits the bacteria *Xylella fastidiosa* Wells et al. (Dellapé et al. 2016), causal agent of the Citrus variegated chlorosis, that affects several commercial species of *Citrus*, and other diseases in plants such as grape vines, almonds, olives, etc. The bacterium is acquired by the plants through the xylem-sap feeding behavior of these insects (Esteves et al. 2020; Coletta-Filho et al. 2020).

One of the most common natural enemies associated with *T. rubromarginata* is the solitary egg parasitoid *Cosmocomoidea annulicornis* (Ogloblin) (Hymenoptera: Mymaridae), that attacks eggs of this sharpshooter laid on very distant host plants taxa, such as *Citrus* spp. (Rutaceae) and Johnsongrass (*S. halepense*) (Poaceae) (Virla et al. 2019). It is widely distributed along South America (Argentina, Brazil, Ecuador and Uruguay) (Jones et al. 2005; Logarzo et al. 2005; Virla et al. 2019, Manzano et al. 2021) and has also been reported parasitizing eggs of other cicadellid species such as *Tretogonia notatifrons* Melichar and *Oncometopia tucumana* Schröder (Virla et al. 2008; Triapitsyn et al. 2010; Dellapé 2013), both polyphagous proconiini sharpshooters that feed on several host plant species (mainly *Citrus* spp.) (Virla et al. 2008; Dellapé and Paradell 2013; Dellapé et al. 2013; Keil and Lozada 2020).

VOCs released by plants upon herbivore attack differ according to the type of herbivore, its feeding guild and the plant species (Turlings et al. 1993; Paré and Tumlinson 1999), and they influence both the behavioral and foraging responses of parasitoids. In this regard, several studies address the variations in the odor blend composition between cultivars of the same plant species and how these affect parasitoid behavior (Gouinguéné et al. 2001; Gols et al. 2011; Degen et al. 2012; Coll Aráoz et al. 2020). Nonetheless, little attention has been given to how the host searching behavior of a parasitoid attacking a polyphagous herbivore is affected by odors emitted by different host plant taxa. Given the wide range of host plants used by *T. rubromarginata* for feeding and oviposition, even in distant plant families such as Rutaceae and Poaceae, we hypothesized that the egg parasitoid *C. annulicornis* is able to identify common cues to locate its host in distant plant families. Therefore, the aims of this study were 1) to study the behavioral response of *C. annulicornis* females to odors emitted by two taxonomically distant host plant species that coexist in the same agroecosystem, citrus and johnsongrass, after feeding and oviposition by *T. rubromarginata* and 2) to identify common traits in the volatile compound emissions by these distant plant species in response to herbivory that could guide the parasitoid toward its host.

Methods And Materials

Plants and Insect Colonies. Potted plants (350 ml) of citrus rootstock Swingle citrumelo 75 ab variety (*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.), hereafter referred to as “citrus”, were used for assays. Plants were provided by “Vivero citrus” (Lules, Tucumán, Argentina). *Sorghum halepense* plants, hereafter referred as “Johnsongrass”, were obtained by sowing fresh rhizomes collected from a field population in potted soil (3000 ml pots). All plants were maintained at 20–25°C, 65–85% RH under natural photoperiod conditions in a greenhouse inside cages covered with “voile” fabric to avoid exposure to wild insects until they were used for bioassays.

Adults of *T. rubromarginata* were collected in a weedy field in San Miguel de Tucumán, Tucumán Province, Argentina (S 26°48'36"- W 65°14'27", elevation 465 m) using an entomological net. The *C. annulicornis* colony was established in the laboratory from *T. rubromarginata* sentinel eggs exposed in citrus trees near the weedy field described above. After exposure to parasitoids, leaves carrying host eggs were removed from the trees and kept in the laboratory until parasitoid emergence. After initial identification of wasps, two different colonies were maintained under laboratory conditions ($25 \pm 3^\circ\text{C}$, 30–45% RH and L14:10D photoperiod), one on eggs of *T. rubromarginata* laid in citrus leaves and another on host eggs laid in Johnsongrass.

Plant Treatments. Plants (citrus and Johnsongrass) used for every assay were subjected to the following treatments: (1) undamaged plants with no previous contact with herbivores. Plants were maintained inside cages as described above isolated before assays. (2) Plants with feeding damage by *T. rubromarginata*. For this, 15–20 males were allowed to feed on potted plants inside “voile” fabric bags for 24 h prior to the behavioral tests or volatile collection. In this treatment, females were not included to avoid oviposition. (3) Plants with host eggs; the same procedure described in treatment 2 was followed, but with 15–20 female sharpshooters instead of males, that fed and oviposited on plants. Only plants carrying more than five host egg masses were used for the assays. (4) Plants carrying host eggs parasitized by *C. annulicornis* females; after exposure of the plants to females of *T. rubromarginata* (equal to treatment 3), host egg masses were exposed to parasitism by *C. annulicornis* females. Each leaf carrying a host egg mass was placed inside a plastic round cage (2.5 cm high x 8.7 cm diam.) without cutting them off from the plants, and with three female parasitoids that were allowed to parasitize for 24 h. Only eggs masses with attack rates higher than 70% were used in the assays.

Olfactometer Assays. Dual choice trials were carried out in order to assess the effect of plant volatiles on the behavioral response of *C. annulicornis* females. Tests were performed using an olfactometer consisting of a Y-tube glass with a central arm (20 cm x 1.5 cm \emptyset) and two side arms (18 cm x 1.5 cm \emptyset). Each side arm was connected to glass recipients hermetically sealed containing the odor source. Airflow (150 ml/min) was pumped, filtered with activated charcoal and humidified through the system before reaching the glass Y-tube. The trials were conducted from \approx 09:00 h to 13:00 h (2.5 to 6.5 h after sunrise) in a chamber under laboratory conditions ($25 \pm 3^\circ\text{C}$, 30–45% RH). The olfactometer was illuminated solely from above with led tube lights (\approx 350 lux) to reduce lighting effect on female parasitoid behavior.

In each trial one newly emerged (< 12 h) naïve *C. annulicornis* female was released in the open end of the central arm of the olfactometer. A choice was recorded after the females crossed an arbitrary decision line close to the beginning of the odor source recipients in each side arm and stayed there for a minute. If a female remained in the central arm for more than 10 min after the release, its behavior was classified as “no-choice”.

Each female was tested only once. In addition, to reduce any asymmetrical bias in the experimental design, the odor sources and their position in the olfactometer were exchanged after 5 females were tested. The Y-tube was cleaned with 96° ethylic alcohol and rotated 180° after each trial. The following

choices were evaluated with the two plant species: (1) clean air vs. undamaged plants (control treatment); (2) undamaged plants vs. plants with feeding damage; (3) undamaged plants vs. plants with host eggs; (4) plants with host eggs vs. plants with parasitized host eggs; and (5) citrus plants with host eggs vs. Johnsongrass plants with host eggs. All plants containing eggs used in the assays had similar quantities of host eggs.

At least 35 females were tested in each assay. Females used for treatments 1–4 were selected either from the colony maintained in citrus or from the Johnsongrass colony, according to the plant species treatment of the assay, while for treatment 5, females were randomly selected from either colony.

Volatile Collection and Analysis. Plant volatile collection was carried out in an isolated room at $25 \pm 3^\circ\text{C}$ and 30–45% RH using the headspace collection technique. The aerial parts of the plants (citrus or Johnsongrass) were hermetically enclosed in volatile-emitting-inert oven plastic bags. The bags had two inlets, one for a Teflon hose that pumped charcoal- filtered air through the plant material inside the bag and another for a second hose that pulled the air by a suction pump at a constant rate of 1 L per min. The Teflon hose connected to the suction pump was attached to a volatile collection trap (30 mg HayeSep Q) where plant volatiles were collected. The system was illuminated from above with a led tube system (≈ 920 lux). System blanks consisted of oven plastic bags without plant material. Up to 6 replicates were performed for each treatment (described above). Volatiles were collected from citrus plants for a period of 3 h between 08:00 h to 11:00 h, while for system blanks and Johnsongrass plants, volatile collections lasted 6 h, between 08:00 h to 14:00 h. The difference in the duration of sample collection was because Johnsongrass volatile emission was expected to be lower than that of citrus plants. After each sampling period, the traps were removed from the apparatus and eluted using dichloromethane (150 μl) containing dodecane (5 ng/ml) as internal standard.

Coupled gas chromatography-mass spectrometry (GC/MS) (Agilent 7890A instrument coupled to Agilent 5977 selective mass detector) with a DB5MS capillary column (0.25-mm i.d., film thickness 0.25 μm) was used to analyze volatile samples, which were injected in a splitless mode at 240°C . Helium (0.75 ml per min) was used as carrier gas. The column temperature was held at 35°C for 1 min, subsequently increased at a rate of 5°C per min until it reached 100°C , and then increased 12°C per min until 230°C and maintained for 10 min. Commercial mass spectra library (NIST 1999) and published data (Adams 2007) were used for identification of compounds. In addition, the kovats retention index on a DB5MS column, the retention times and mass spectrum of each compound were compared with authentic standards (Symrise) of known compounds and the alkane series. The AMDIS software (Automated Mass Spectral Deconvolution and Identification System) and the report obtained from TIC were used to quantify the detected volatiles based on the comparison of their peak area with that of the internal standard (dodecane).

Statistical Analysis. Significant differences in female choices in olfactometer assays were analyzed by means of the Exact binomial test ($\alpha = 0.05$). Non-responsive females were not included in this analysis. Average values of volatile compounds subjected to different experimental treatments were compared

using one-way ANOVA. Bartlett's and Shapiro-Wilk tests were performed to test for data normality and variance homogeneity. Post hoc comparisons of the means were performed by Tukey honestly significant difference (HSD). If data did not follow ANOVA requirements, and failed to be log-transformed, Kruskal-Wallis tests were carried out and post hoc Dunn's multiple comparison tests were performed. Compounds that were only present in two of the treatments were analyzed by the unpaired t-test or by Wilcoxon test if t-test assumptions were not met.

A non-metric multidimensional scaling analysis (NMDS), using a Bray-Curtis distance matrix and 5000 iterations, was used to ordinate treatments according to range of VOCs produced by each one. The resultant value of ordination stress for NMDS was below the accepted threshold of 0.20 (Dexter et al. 2018) and, in the case of citrus it was equal to 0.097, while in Johnsongrass it was equal to 0.111. All data analyses were performed using R statistical software version 4.1.0 (R core team 2021).

Results

Olfactometer Assays. The results of the dual choice olfactometer tests showed that *C. annulicornis* females were significantly attracted to host eggs oviposited in both plant species, citrus and Johnsongrass (Exact binomial test, $P = 0.005$ and $P = 0.01$, respectively). Females did not discriminate between undamaged plants, with no previous contact with the herbivore (undamaged citrus or Johnsongrass plants) vs. clean air (Exact binomial test, $P = 0.39$ and $P = 0.86$). Interestingly, even in the absence of host eggs, females significantly chose citrus plants with feeding damage performed by herbivore males (Exact binomial test, $P = 0.03$) over undamaged plants. However, in the case of Johnsongrass plants, females did not make a significant choice between plants with feeding damage and no host eggs when contrasted against undamaged plants (Exact binomial test, $P = 0.51$). Considering previous parasitism by conspecifics, females did not discriminate between healthy eggs of *T. rubromarginata* and host eggs previously parasitized laid either on citrus (Exact binomial test, $P = 0.55$) or Johnsongrass plants (Exact binomial test, $P = 0.15$) (Fig. 1A). Lastly, there was no significant difference in the preference of females when given a choice between host eggs laid on citrus or Johnsongrass (Exact binomial test, $P = 0.47$) (Fig. 1B).

Volatile Collection and Analysis. The headspace sampling of citrus and Johnsongrass plants allowed the characterization of VOCs emitted constitutively by undamaged plants and those under herbivory damage, either by feeding alone, feeding + host eggs and with feeding + parasitized eggs. The two plant species differed greatly in their VOCs profiles. A total of 36 and 22 VOCs were identified from the samples collected from citrus and Johnsongrass plants, respectively (Table 1). Several compound classes were detected in the analysis: aromatic and aliphatic hydrocarbons, mono- and sesquiterpenes, and organic acids. Twelve common volatiles were found between the two plant species (acetophenone, benzene, 1-ethyl-2-methyl-, cumene, decanal, β -caryophyllene, limonene, indole, methyl anthranilate, sabinene, terpinene, β -farnesene and β -pinene), two of which were detected in herbivory induced treatments (β -caryophyllene and indole). In damaged citrus plants with host eggs, β -caryophyllene emissions exceeded

indole by tenfold, while the proportion of β -caryophyllene and indole in Johnsongrass was similar in the same treatment.

Table 1

Statistical results of constitutive and herbivore-induced VOCs (mean \pm SE in ng h⁻¹) emitted by citrus and Johnsongrass plants. The Kovats retention index (RI) and chemical type of each compound is given. Different letters within the same row indicate significant differences ($P < 0.05$). Compounds authenticated with commercial standards are indicated by *.

Compound	Type	RI	UP	PFD	PHE	PPHE
Citrus			N = 6	N = 6	N = 4	N = 4
Unknown 1		865	—	—	1.91 \pm 0.83 a	1.36 \pm 1.09 a
Unknown 2		926	—	—	—	2.45 \pm 1.15
Benzaldehyde	Aromatic	954	—	4.07 \pm 1.06	—	—
Benzene, 1-ethyl-2-methyl-	Aromatic	969	6.42 \pm 3.16 a	8 \pm 2.68 a	1.14 \pm 0.90 a	—
Sabinene	Aromatic	980	14.58 \pm 7.03 a	7.86 \pm 5.99 a	23.9 \pm 1.14 a	6.36 \pm 6.36 a
β -Pinene	Monoterpene	983	6.84 \pm 6.84 a	3.96 \pm 3.96 a	17.65 \pm 10.29 a	—
Cumene	Aromatic	1000	4 \pm 1.44 a	2.41 \pm 0.78 a	—	—
o-Cymene	Aromatic	1016	1.06 \pm 1.06 a	1.62 \pm 1.04 a	—	0.76 \pm 0.76 a
Terpinene*	Monoterpene	1022	1.44 \pm 0.74	—	—	—
β -Ocimene*	Monoterpene	1030	2.89 \pm 1.71 a	7.42 \pm 5.18 a	23.57 \pm 5.79 b	53.19 \pm 23.74 b
Limonene*	Monoterpene	1034	172.85 \pm 66.11 a	120.95 \pm 74.21 a	349.79 \pm 53.34 a	153.4 \pm 73.30 a
Benzene, 1,2-diethyl-	Aromatic	1042	42.65 \pm 33.96 a	81.89 \pm 30.52 a	—	—
UP: Undamaged plants						
PFD: Plants with feeding damage						
PHE: Plants with host eggs						
PPHE: Plants with parasitized host eggs						
EIMS (70 eV) of Unknown 1 (obtained by GC-MS): m/z (rel. int. %) 86 (75), 84 (99), 70 (79), 55 (55), 49 (90), 43 (100)						
EIMS of Unknown 2: 93 (90), 86 (62), 84 (100), 49 (95)						

Compound	Type	RI	UP	PFD	PHE	PPHE
3-Carene	Aromatic	1049	3.79 ± 1.41 a	–	–	–
1-Ethyl-2,3-dimethylbenzene	Aromatic	1066	0.61 ± 0.30 a	–	–	–
Acetophenone	Aromatic	1070	–	5.8 ± 4.56 a	2.04 ± 2.04 a	12.56 ± 10.42 a
Undecane	Aliphatic	1087	8.06 ± 2.56 a	3.82 ± 1.32 ab	0.15 ± 0.15 b	–
Allo-Ocimene	Monoterpene	1117	0.04 ± 0.04 a	–	0.19 ± 0.19 a	1.59 ± 0.93 a
Citronellal	Monoterpene	1144	8.82 ± 7.47 a	–	16.14 ± 6.81 a	7.27 ± 4.37 a
Acetic acid, phenylmethyl ester	Aromatic	1145	1.05 ± 0.73 a	1.61 ± 0.72 a	–	–
Methyl salicylate	Aromatic	1182	–	–	0.30 ± 0.18 a	2.24 ± 0.77 a
m-Ethylacetophenone	Aromatic	1252	2.06 ± 1.74 a	2.73 ± 0.94 a	–	–
Decanal	Aliphatic	1254	1.13 ± 0.56 a	1.06 ± 0.47 a	0.27 ± 0.27 a	1.1 ± 0.69 a
p-Ethylacetophenone	Aromatic	1270	2.12 ± 1.86 a	3.03 ± 1.19 a	–	–
Indole	Aromatic	1302	–	–	1.11 ± 1.11 a	1.57 ± 0.56 a
δ-Elemene	Sesquiterpene	1325	0.73 ± 0.59 a	–	1.21 ± 0.74 a	0.48 ± 0.29 a
β-Elemene	Sesquiterpene	1385	3.61 ± 2.5 a	1.25 ± 1.25 a	6.44 ± 2.82 a	3.63 ± 1.52 a

UP: Undamaged plants

PFD: Plants with feeding damage

PHE: Plants with host eggs

PPHE: Plants with parasitized host eggs

EIMS (70 eV) of Unknown 1 (obtained by GC–MS): m/z (rel. int. %) 86 (75), 84 (99), 70 (79), 55 (55), 49 (90), 43 (100)

EIMS of Unknown 2: 93 (90), 86 (62), 84 (100), 49 (95)

Compound	Type	RI	UP	PFD	PHE	PPHE
Methyl anthranilate	Aromatic	1425	5.19 ± 2.97 a	3.56 ± 2.45 a	17.72 ± 8.79 a	9.12 ± 4.56 a
γ-Elemene	Sesquiterpene	1425	1.72 ± 1.41 a	1.03 ± 1.03 a	3.72 ± 1.56 a	2.15 ± 0.72 a
β-Caryophyllene*	Sesquiterpene	1441	4.31 ± 3.27 a	2.85 ± 2.19 a	10.71 ± 1.55 b	5.39 ± 0.85 ab
Humulene*	Sesquiterpene	1451	0.9 ± 0.65 a	—	1.22 ± 0.70 a	0.39 ± 0.39 a
β-Farnesene	Sesquiterpene	1472	1.12 ± 0.81 a	—	1.04 ± 1.04 a	0.95 ± 0.55 a
β-Cubebene*	Sesquiterpene	1479	2.83 ± 1.63 ab	1.47 ± 1.02 b	6.24 ± 1.70 a	3.60 ± 0.96 ab
Patchoulene	Sesquiterpene	1494	1 ± 0.70 a	—	0.88 ± 0.52 a	0.3 ± 0.17 a
α-Farnesene	Sesquiterpene	1498	—	—	4.62 ± 1.60	12.35 ± 11.06 a
δ-Cadinene	Sesquiterpene	1516	0.81 ± 0.57 a	—	0.36 ± 0.39 a	0.77 ± 0.27 a
Butyl citrate	Organic acid	2123	17.52 ± 11.60 a	33.78 ± 10.89 a	—	—
Total monoterpenes			191.44	132.33	407.34	215.45
Total sesquiterpenes			17.18	6.6	31.46	30.01
Total aromatics			83.53	118.51	46.21	33.04
Total aliphatic			9.19	4.88	0.42	1.10
Total organic acids			17.52	33.78	—	—
Total VOCs			320.86 ± 74.52a	295.66 ± 55.83 a	482.92 ± 42.79a	283.06 ± 67.35a

UP: Undamaged plants

PFD: Plants with feeding damage

PHE: Plants with host eggs

PPHE: Plants with parasitized host eggs

EIMS (70 eV) of Unknown 1 (obtained by GC-MS): m/z (rel. int. %) 86 (75), 84 (99), 70 (79), 55 (55), 49 (90), 43 (100)

EIMS of Unknown 2: 93 (90), 86 (62), 84 (100), 49 (95)

Compound	Type	RI	UP	PFD	PHE	PPHE
Johnsongrass			N = 5	N = 2	N = 6	N = 3
Ethylbenzene	Aromatic	882	7.31 ± 3.57 a	–	8.59 ± 3.12 a	2.07 ± 2.07 a
α-Pinene*	Monoterpene	956	12.06 ± 1.96 a	16.39 ± 0.78 a	10.79 ± 1.74 a	11.48 ± 3.14 a
Benzene, 1-ethyl-2-methyl-	Aromatic	969	5.95 ± 1.83 a	11 ± 0.02 a	5.16 ± 1.51 a	2.28 ± 2.228 a
Sabinene	Monoterpene	980	1.87 ± 0.55 a	2.77 ± 0.06 a	2.13 ± 1.09 a	1.53 ± 0.81 a
β-Pinene	Monoterpene	989	2.73 ± 0.84 a	4.93 ± 0.11 a	2.70 ± 0.39 a	2.62 ± 0.68 a
Cumene	Aromatic	1000	–	2.86 ± 0.02 a	0.46 ± 0.29 a	0.55 ± 0.05 a
Decane	Alkane	1021	1.47 ± 0.65 a	3.26 ± 0.34 a	1.01 ± 0.52 a	2.94 ± 0.56 a
Terpinene*	Monoterpene	1022	–	2.78 ± 0.09	–	–
α-Phellandrene	Monoterpene	1025	29.89 ± 5.64 a	29.79 ± 0.73 a	6.56 ± 1.61 b	6.49 ± 2.16 b
Limonene*	Monoterpene	1034	27.06 ± 5.12 a	31.75 ± 2.23 a	36.10 ± 12.95 a	11.83 ± 3.83 a
Hemellitrol	Aromatic	1043	–	2.85 ± 0.07 a	0.98 ± 0.49 b	–
p-Cymene	Aromatic	1046	11.96 ± 2.18 a	16.1 ± 0.6 a	8.24 ± 1.20 ab	3.47 ± 2.32 b
Eucalyptol	Monoterpene	1053	1.79 ± 0.88 a	3.94 ± 0.02 a	1.42 ± 0.56 a	1.33 ± 0.68 a

UP: Undamaged plants
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EIMS (70 eV) of Unknown 1 (obtained by GC–MS): m/z (rel. int. %) 86 (75), 84 (99), 70 (79), 55 (55), 49 (90), 43 (100)
EIMS of Unknown 2: 93 (90), 86 (62), 84 (100), 49 (95)

Compound	Type	RI	UP	PFD	PHE	PPHE
Acetophenone	Aromatic	1070	3.4 ± 2.09 a	3.34 ± 0.57 a	3.35 ± 2.36 a	3.53 ± 1.58 a
Nonanal	Aliphatic	1126	–	2.62 ± 2.62 a	3.75 ± 0.61 a	1.91 ± 0.96 a
1,2-benzisothiazole	Aromatic	1249	–	2.76 ± 0.21 a	0.52 ± 0.34 b	–
Decanal	Aliphatic	1254	–	2.96 ± 0.34 a	2.16 ± 0.57 a	2.43 ± 0.06 a
Indole	Aromatic	1302	–	2.61 ± 0.30 a	1.94 ± 0.73 a	0.71 ± 0.71 a
Methyl anthranilate	Organic acid	1425	–	–	1.22 ± 0.88	–
β-Caryophyllene*	Sesquiterpene	1441	–	–	1.30 ± 0.51	–
β-Farnesene	Sesquiterpene	1472	–	2.66 ± 2.66 a	2.32 ± 1.79 a	0.53 ± 0.53 a
α-Bergamotene	Sesquiterpene	1479	–	1.89 ± 1.89 a	3.67 ± 1.91 a	0.62 ± 0.62 a
Total monoterpenes			75.40	92.35	59.70	35.28
Total sesquiterpenes			–	4.55	7.29	1.15
Total aromatics			28.62	41.52	29.24	12.61
Total aliphatic			1.47	8.84	6.92	7.28
Total organic acids			–	–	1.22	–
Total VOCs			105.38 ± 17.61a	149.61 ± 7.92a	104.49 ± 23.75a	57.05 ± 8.96a
UP: Undamaged plants						
PFD: Plants with feeding damage						
PHE: Plants with host eggs						
PPHE: Plants with parasitized host eggs						
EIMS (70 eV) of Unknown 1 (obtained by GC-MS): m/z (rel. int. %) 86 (75), 84 (99), 70 (79), 55 (55), 49 (90), 43 (100)						
EIMS of Unknown 2: 93 (90), 86 (62), 84 (100), 49 (95)						

The VOCs emitted by citrus plants after feeding and oviposition damage included indole, methyl salicylate, β-caryophyllene, acetophenone, benzaldehyde, undecane, α-farnesene and two unknown

compounds. Few compounds present in the headspace of undamaged plants were either emitted in lower quantities (undecane: $F = 7.24$; $df = 2$; $P = 0.007$) or absent in plants with host eggs, either healthy or parasitized (cumene, m-ethylacetophenone and p-ethylacetophenone).

On the other hand, Johnsongrass plants with herbivory damage emitted 1,2-benzisothiazole, indole, anthranilic acid, hemellitol, β -caryophyllene, decanal, nonanal, terpinene, α -bergamotene, α -phellandrene and β -farnesene, which were absent in the headspace of undamaged plants. The only compound detected in significantly lower amounts in Johnsongrass plants with host eggs either healthy or parasitized compared to undamaged plants and plants with feeding damage alone was α -phellandrene ($F = 8.91$; $df = 3$; $P = 0.002$).

The NMDS plots showed different degrees of ordination for each of the analyzed treatments (Fig. 2). For citrus plants, a total of 21 VOCs were significantly correlated with both NMDS axes, while for Johnsongrass plants, eight VOCs correlated significantly with the NMDS axes. Results of both citrus and Johnsongrass analysis showed that plants containing healthy and parasitized *T. rubromarginata* eggs were dissimilar to undamaged plants and to plants with damage by sharpshooter males. In the case of citrus, undamaged and plants with feeding damage were more closely related to each other than plants carrying healthy and parasitized sharpshooter eggs (Fig. 2a). In addition, oviposited Johnsongrass plants (with healthy and parasitized eggs) were closer together than healthy and feeding damaged plants (Fig. 2b).

Discussion

Females of the egg parasitoid *Cosmocomoidea annulicornis* were able to detect host eggs irrespective of the plant that held them, relying only on common volatile cues. However, they did not discriminate between healthy and parasitized eggs by conspecifics.

The headspace analysis of citrus and Johnsongrass plants with host eggs revealed very few similarities in the blend's composition. Among the compounds detected, only indole and β -caryophyllene increased in both plant species with oviposition damage. Indole is an aromatic compound that is specifically emitted after herbivore damage in several plants (Erb et al. 2015), although its ecological role as an attractant of natural enemies is uncertain, since some parasitoid species are known to be attracted (James 2005) and others repelled by this volatile (D'Alessandro et al. 2006; Block et al. 2018). Also, it can have a dual effect, as an attractant and a repellent (Ye et al. 2018), which shows that there may not be a straightforward response (Zhuang et al. 2012). β -Caryophyllene is also known to be released during herbivore attack and to act as an attractant for several parasitoids in plants such as maize (Kollner et al. 2008; Tamiru et al. 2017), cotton (Loughrin et al. 1994), beans (Colazza et al. 2004a, b) and elm (Büchel et al. 2011). Some parasitoids find their hosts in distant plant species exploiting similarities in the relative proportions of herbivory induced VOCs in each blend (De Moraes et al. 1998), however, we could not find such a common relation in the ratios between compounds found in both infested plants (indole and caryophyllene).

Based on plant odor alone, there were no signature volatiles or volatile blends that could trustfully indicate the presence of the host herbivore. However, common herbivory induced plant VOCs present in the odor profiles could induce long range foraging decisions, and once on the plant, other sensory inputs could also be very important, particularly visual and physical cues (Aquino et al. 2012; Michereff et al. 2016).

In agroecosystems, parasitoids must interpret very complex chemical environments while searching for their hosts. Herbivory induced VOCs are elicited by conserved plant signaling metabolic pathways (Huffaker et al. 2013). Although terpene synthases are diversified throughout the plant kingdom (Chen et al. 2011), a few induced VOCs are usually released by most plant species, irrespective of their taxonomic affinities (Clavijo McCormick et al. 2012). For example, under herbivore attack many plant species emit the terpenoids (E)- β -ocimene, linalool, α -farnesene, β -caryophyllene and 4,8-dimethyl-1,3,7-nonatriene, although the composition of the blend and relative amounts of compounds may vary among species and even among cultivars or varieties of the same species (Degen et al. 2004; Clavijo McCormick et al. 2012; Coll Aráoz et al. 2020).

Besides indole and β -caryophyllene, citrus plants infested with host eggs displayed differences in the emissions of β -ocimene, undecane, butyl citrate and two unknown compounds. Although D-limonene was the dominant compound detected in citrus plants, there were no variations in the amounts released after herbivore damage. Contrary to this result, Mohammed et al. (2020) found that D-limonene levels sharply increased in several *Citrus* species after infestation with the pest *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), and that its parasitoid, *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), was attracted to this volatile combined with β -Ocimene. This last compound is released by grapevines plants after feeding and oviposition of the proconiini sharpshooter *Homalodisca vitripennis* (Germar) and it affects the foraging behavior of the egg parasitoid *Cosmocomoidea ashmeadi* Girault (formerly *Gonatocerus*), which positively responded to this volatile when it was combined with α -farnesene (Krugner et al. 2014).

Comparatively, Johnsongrass plants under oviposition damage released lower amounts of α -phellandrene and hemellitol than undamaged plants. Takemoto et al. (2015) reported that broad bean plant emission of α -phellandrene under aphid attack was attractive for the parasitic wasp *Aphidius ervi* Haliday (Hymenoptera: Braconidae).

The attraction of wasps to volatiles induced by the feeding of adult sharpshooters was subject to the attacked host plant species. Parasitoid females were significantly attracted to citrus plants with feeding damage performed by males, while no effect of this treatment was observed in Johnsongrass plants. The most noticeable difference between the two host plant species under feeding damage was the presence of benzaldehyde in citrus plants. This compound is known to be emitted by other plant species and has proven to be an attractant to the insect pest *Sitona humeralis* Stephens (Coleoptera: Curculionidae) (Lohonyai et al. 2019), the aphid *Rhopalosiphum padi* (L.) (Park et al. 2000) and to wild Trichogrammatidae parasitoids in a field study (Simpson et al. 2011). It is possible that other host related chemical cues such as salivary secretions and faeces, would also be important for parasitoids attacking

insects with overlapping generations like *T. rubromarginata* (Freytag and Sharkey 2002), as environments with adults presence are likely to contain eggs (Vet and Dick 1992; Peñaflor et al. 2011).

Headspace samples of plants with parasitized host eggs displayed similar compounds to those detected in plants with non-attacked host eggs, although a number of volatiles present in plants with host eggs were no longer detected in the parasitized samples (citrus: β -Pinene, Undecane and Benzene, 1-ethyl-2-methyl; Johnsongrass: hemellitol, β -caryophyllene, 1,2-benzisothiazole and methyl anthranilate). It is noteworthy that the VOC "Unknown 2" was only detected in citrus samples with eggs parasitized by *C. annulicornis*.

The fact that *C. annulicornis* females did not discriminate between healthy and parasitized host eggs during behavioral tests suggests that the presence of a conspecific developing larvae does not influence the attractiveness of the host eggs, because females were not able to detect the differences in the volatile blends. There are no records of superparasitism in this species (Manzano et al. 2021) so it is likely that females rely on probing behavior before ovipositing eggs parasitized by conspecifics. There are several studies demonstrating that for egg parasitoids, probing and subsequent egg marking by females are frequent behaviors that condition host recognition and discrimination of previously attacked eggs (Weber et al. 1995; Keinan et al. 2012).

NMDS analysis highlighted that regardless of the host plant species, plants differentially emitted volatiles after oviposition damage (including healthy and parasitized eggs) compared to untreated and plants with feeding damage alone. Interestingly, despite the qualitative variation in compounds released among different treatments, there were no differences in the total amount of volatiles emitted constitutively and under herbivore damage in the two host plant species analyzed.

It has been generally assumed that natural enemies use herbivory induced plant volatiles to find the plant species where their hosts are likely to be present. For egg parasitoids this seems to be especially true, since insects' eggs only emit small quantities of volatiles (Fatouros et al. 2008). As demonstrated in the olfactometer assays, *C. annulicornis* females rely on chemical cues to find their hosts, however, this attraction toward plant volatiles would not necessarily translate into an increase in the parasitoid's fitness (Clavijo McCormick et al. 2012). Although females might be using volatiles to locate infested plants, there are probably other chemical cues detected at short range that mediate the final decision to oviposit.

Previous studies have shown that plant species influences *C. annulicornis* oviposition rates. Virla et al. (2019) report significantly higher parasitism rates in the field in host eggs laid on citrus plants, compared to those laid on Johnsongrass. Similarly, laboratory studies support this trend (Manzano et al. 2021), demonstrating that fitness of this parasitoid is highly dependent on plant species. However, in olfactometer assays *C. annulicornis* females showed no preference between eggs carried by any of the two host plant species. Common volatiles emitted in response to herbivory would be important for orientation toward the host, but oviposition could be mediated by direct contact cues. It is possible that female final acceptance of hosts is based on other traits besides olfactory cues (Colazza et al. 2009),

such as leaf morphological structures (Virla et al. 2020), contact cues (Iacovone et al. 2016) and host quality (Hofstetter and Raffa 1998, Zhou et al. 2014).

Our investigation contributes to the understanding on how chemical cues emitted by host plants affect the host searching behavior of an egg parasitoid and constitutes a step forward to finding an attractive blend for the parasitoid of this economically important herbivore species. The manipulation of VOCs released by plants in response to herbivory can be a valuable tool for developing new strategies of integrated pest management (IPM) to increase biological control and its cost efficiency.

Declarations

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Conflict of interest. The authors declare that they do not have any conflict of interest associated with this work.

Authors' contributions. All authors contributed to the study conception and design. CM and MVCA wrote the manuscript. CM and PCF carried out the experiments. CM and JGH conducted insect field collections and statistical analyses. ELA, EGV, MVCA and PCF supervised the project, revised the manuscript and secured funding. All authors helped shape the research, analysis and approved the manuscript.

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Figures

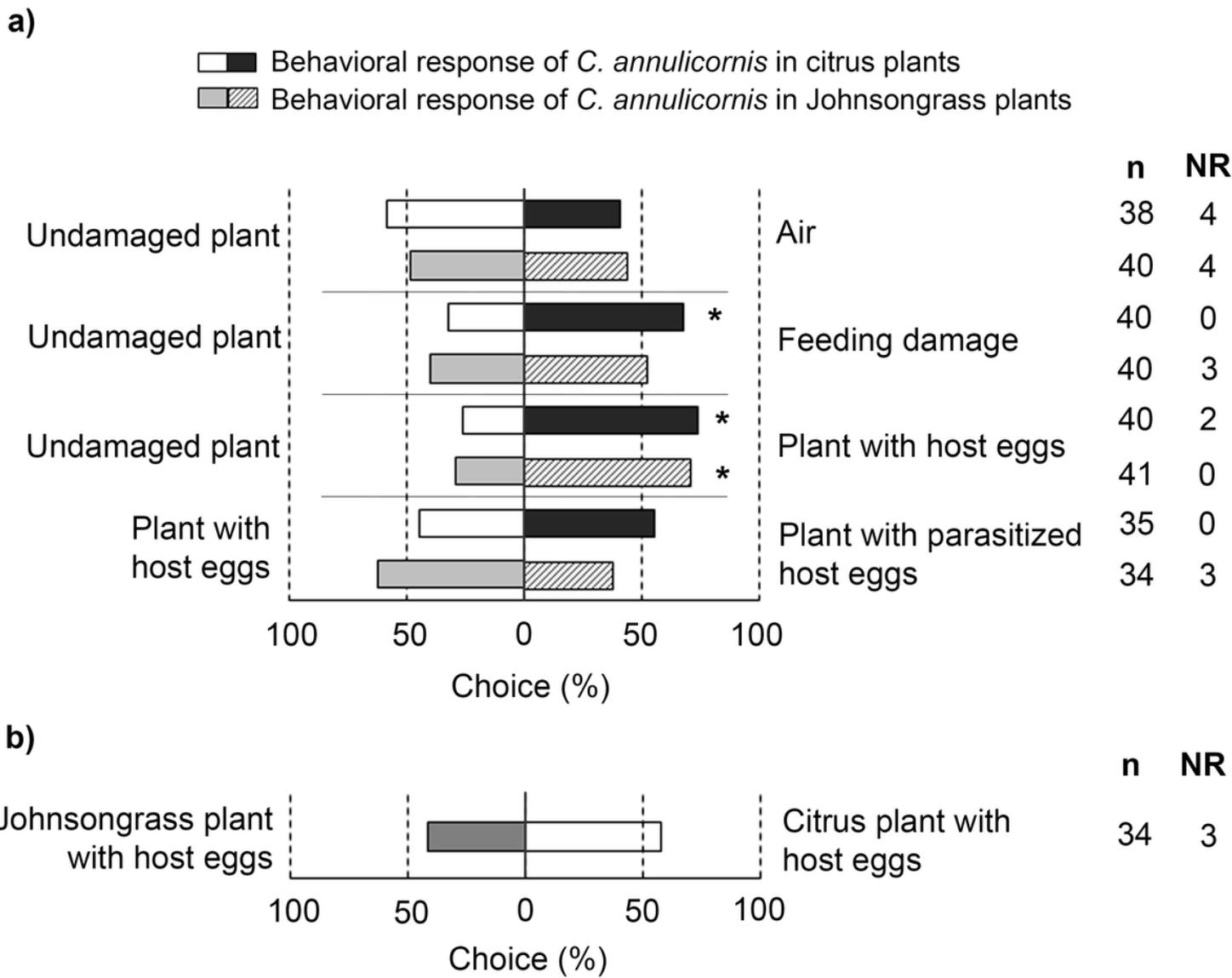


Figure 1

Response of *Cosmocomoidea annulicornis* females tested in olfactometer assays. A) Female dual-choice (%) for volatiles emitted by citrus and Johnsongrass plants under different treatments; B) Female dual-choice (%) between the two plant species carrying eggs of *Tapajosa rubromarginata*. Asterisks denote significant difference between treatments in exact binomial tests ($P < 0.05$). The number of replicates performed for each treatment (n) and the number of non-responsive females (NR) are given on the right side of the chart.

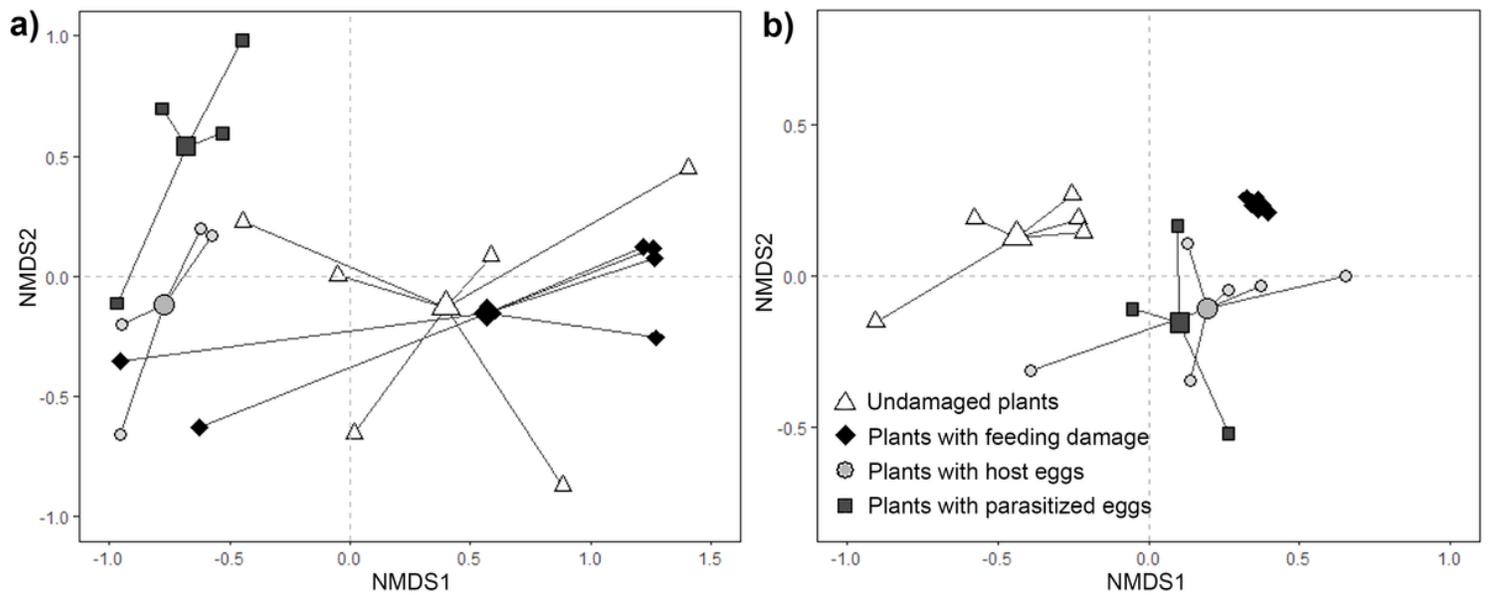


Figure 2

Non-metric multidimensional scaling (NMDS) ordination plot, based on Bray-Curtis distance matrix, for the relative amounts of volatiles released by undamaged and herbivore-damaged plants (feeding and oviposition damage), and plants containing previously parasitized eggs of *T. rubromarginata* on a) citrus; and b) Johnsongrass. The points and the different shapes indicate samples and treatments analyzed. Large-size points denote centroids for each treatment.