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Attractant, sexual competitiveness enhancing and toxic activities of the essential oils from *Baccharis spartioides* and *Schinus polygama* on *Ceratitis capitata* Wiedemann

Flavia Jofré Barud[a], Sandra López[a,b], Alejandro Tapia[a,b], Gabriela E. Feresin[a,b], María Liza López[a,*]

[a] *Instituto de Biotecnología, Facultad de Ingeniería, Universidad Nacional de San Juan, Av. Libertador General San Martín 1109 (O), Capital, CPA: J5400ARL San Juan, Argentina*

[b] *Instituto de Ciencias Básicas, Facultad de Filosofía, Humanidadesy Artes, Universidad Nacional de San Juan, Av. Libertador General San Martín 1109 (O), Capital, CPA: J5400ARL San Juan, Argentina*

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A B S T R A C T

The sterile insect technique is currently used to control the fruit fly pest, *Ceratitis capitata* Wiedemann. The problem with this technique is a decreased sexual competitiveness of the sterile males. We tested the semiochemical properties of essential oils from non-host species as sexual performance enhancers of *C. capitata* as well as the attractant activity and toxic effect of the oils on adults. By GC–MS analysis, thirty-seven compounds were identified in the essential oil from *Schinus polygama* (Anacardiaceae) with relative proportions of the compounds up to 5%. The essential oil from *Baccharis spartioides* (Asteraceae) presented three monoterpenes hydrocarbon dominating the chemical profile. Prior exposure of males to both essential oils increased the sexual competitiveness compared to untreated males in mating trials. In olfactometry tests, males were slightly attracted by the oil of *S. polygama*, and the essential oil of *B. spartioides* significantly attracted the females. We postulate these two aromatic species as new sources of essential oils that can enhance the sexual performance of sterile medfly males used in SIT programs. Moreover, the attractant and toxic properties of the oils suggest they can be candidate substances for lure-and-kill technologies.

1. Introduction

Currently, many strategies to achieve acceptable population levels of insect pests are based on modifying the insect behavior. Technologies such as lure-and-kill, push-pull systems, and sexual disruption are based in the need of insects to use chemical signals to find mates; locate food sources or oviposition sites (Camelo et al., 2007; Cook et al., 2007; Witzgall et al., 2008).

Plant semiochemicals elicit diverse behavioral responses in insects. In some cases, sexual signaling and pheromone emission begin in the presence of host plant volatiles (Papadopoulos et al., 2006); while in other cases volatiles from host plants synergistically enhance the response of a given sex to the sex pheromones of members of the other sex (Schmidt-Büsser et al., 2009; Reddy and Guerrero, 2004). In other insects, host plant compounds are ingested and used as sex pheromones or as sex pheromone precursors (Nishida et al., 2009; Kah-Wei Hee and Keng-Hong, 2006). In addition, plant volatiles can attract predators or parasitoids of the attacking species after herbivory injury (Reddy and Guerrero, 2004). Accordingly, semiochemicals implicated in the chemical ecology of insects are increasingly being used to develop sustainable tools for the pest control.

The worldwide pest species *Ceratitis capitata* (Wiedemann), the "Mediterranean fruit fly" (Diptera, Teprhitidae) is a multivoltine and highly polyphagous species that attacks the ripening fruit of numerous commercially important fruit crops (Liquido et al., 1991). The species causes large fruit losses, both by direct damage to the fruits or by the quarantine precautionary measures imposed by pest-free countries (Malavasi et al., 1994; Ovruski et al., 1999). The sterile insect technique (SIT) is used worldwide to control the Mediterranean fruit fly, but its effectiveness is limited by low sexual competitiveness of mass-reared males relative to wild males (Shelly, 2012). One way to enhance mating performance of sterile males is to expose them before being released in the field to the

* Corresponding author. Tel.: +54 264 4211700; fax: +54 264 4211700.
E-mail address: mllopez@unsj.edu.ar (M.L. López).

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volatiles of ginger root oil, a known source of α-copaene (Shelly et al., 2004).

α-Copaene is a minor component of the essential oils of various plant species including hosts of *C. capitata* such as orange, guava, papaya, and mango (mostly without knowledge on the enantiomeric purity) (Nishida et al., 2000). The (+)-enantiomer of α-copaene is known to be responsible for male attraction, while the (−)-enantiomer is less active (Jacobson et al., 1987; Flath et al., 1994). The compound potentially serves as a chemical cue to facilitate orientation of flies to the rendezvous site for courtship and mating (Nishida et al., 2000). Medfly males feed on host plants or exposed to α-copaene increase the production and release of sexual pheromones and spent more time in pheromone-calling activity (Shelly, 2001; Shelly and Villalobos, 2004). The oil of ginger root containing α-copaene, and the essential oil from the flavedo region of citrus fruits greatly improve the competitiveness of medfly males, both wild and mass reared for the SIT ensuring a greater number of couples (Shelly et al., 2004; Katsoyannos et al., 1997; Papadopoulos et al., 2001; Vera et al., 2013). However, the relative increase in calling activity seems insufficient to explain the magnitude of the increase in mating success. Later, it was suggested that a "perfume" effect could explain the preference of the medfly females to copulate with pre-exposed males to the oils of ginger root (Shelly et al., 2007a). In addition, other citrus compounds such as limonene, geraniol, α-pinene, β-myrcene and linalool can have a similar effect (Kouloussis et al., 2013). Thus, such mono- and sesquiterpenes might be naturally implicated in the mating of medflies.

Monoterpenes and sesquiterpenes are ubiquitous in essential oils of many wild aromatic species. *Baccharis* is the largest genus in the Asteraceae with over 500 species. It is a genus native to America reputed by the bioactivity of their essential oils including antimicrobial (Oliva et al., 2007), insecticidal (Sosa et al., 2012; Kurdelas et al., 2012) and nematicidal activities (Sosa et al., 2012). Another native genus to America is *Schinus* from the Anacardiaceae. The essential oils or organic extracts of *Schinus* species have been reported to possess repellent and toxic activities on insect pests (Chiffelle et al., 2013; Descamps et al., 2011; Abdel-Sattar et al., 2010) and disease vectors (Ferrero et al., 2006). However, these essential oils have never been evaluated beyond the conventional biocidal or repellent effect. Given their high bioactivity, they should be explored as possible attractants or sexual enhancers of *C. capitata*. In all, our objective was to evaluate the effect of the essential oils of *B. spartioides* and *S. polygama* as attractants and sexual enhancers of sterile males of *C. capitata* as well as toxic properties of the oils on both sexes.

2. Methods and materials

2.1. Plant material

Aerial parts of *Baccharis spartioides* (Hook. &Arn. ex DC.) J. Remy (Asteraceae) and *Schinus polygama* (Cav.) Cabrera (Anacardiaceae) were collected in summer of 2010, in Angaco (31°12′0″ S–68°7′60″ W) and Bauchaceta district (30°10′40″ S–69°27′30″ W), province of San Juan, Argentina, respectively.

2.2. Essential oil extraction

Fresh aerial parts (150 g) were subjected to steam distillation for 2 h using a Clevenger type apparatus. The yields were averaged over two independent experiments and calculated according to dry weight of plant material. Essential oils were stored at −18 °C in airtight micro-tubes prior to chemical analysis.

2.3. Chemical analysis

The oils were analyzed by GC-MS. Mass spectra were obtained on an Agilent model 5975C MSD mass spectrometer, coupled directly to an Agilent 7890 A gas chromatograph fitted with a HP-5MS column (30 m long, 0.25 mm i.d., 0.25 μm film thickness). The GC–MS was operated under the following conditions: injector temperature, 250 °C; detector temperature, 280 °C; oven temperature programmed 5 min isothermal at 50 °C, subsequently increased at 4 °C/min to 280 °C, and then held isothermally for 10 min. The carrier gas was helium at 0.9 mL/min, and the ionization voltage was 70 eV. Identification of the components was performed by comparison of their retention index (RI) with reference to a homologous series of n-alkanes (C9–C25), by comparing their mass spectra with those reported in literature and by computer matching with the Wiley 5 and Adams libraries. Quantitative percentage composition was determined from the GC peak areas without correction factors.

2.4. Insects

Sterile males and females of *C. capitata* (*tsl* strain – Vienna 8) were supplied by ProCEM-SENASA (San Juan, Argentina) at the pupal stage (two days before adults' emergence) after irradiation with Cobalt 60 at 120 Gy. Hatched adults were kept in chambers provided with water and artificial diet (sugar: yeast hydrolysate 3:1) *ad libitum*, under controlled conditions of temperature (24 ± 2 °C), relative humidity (50 ± 5%) and light (16 h L/8 h D). Sexes and cohorts were kept apart. Sexes and cohorts were kept apart.

2.5. Attractant activity of the oils on Ceratitis capitata adults

The bioassays were performed in laboratory conditions (24 ± 2 °C, 50 ± 5% RH) in a Y-tube olfactometer. The glass Y-olfactometer (5 cm id, stem 16 cm, branches 10 cm) terminated in threaded-glass joints, and Teflon screw caps were connected to two separate glass vials (250 mL) with Teflon tubing. A tripod held the Y-tube in an inclining position (angle 25° between Y-tube and horizontal plane). An electric pump was used to pump air into the Y-olfactometer. The air stream, moistened and pre-filtered through activated charcoal, was blown into the Y-olfactometer at 120 mL/min with a flow meter (Supelco). The tested material consisted in 1 cm^2 of filter paper treated with 2 μL of acetone containing 5 μg of oil compared with 2 μL of acetone as control. Filter papers were placed in each vial and were discarded after every single trial. Essential oil was applied to a piece of paper 30 min before the first fly was released. For each trial, a single insect was placed on the base of the stem of the Y-tube. Each insect was allowed to explore the Y-tube with no air flow for 2 min. Then, the air stream was activated for 2 min. A fly was considered to have made a first choice when it moved into either arm (visually assessed by a line marked on both arms). The time (s) flies spent in each arm of the device was also recorded. Flies' final choice was the arm they were in at the end of the 2 min experimental period. Control experiments using air *vs* air and solvent *vs* solvent indicated that each arm of the Y-tube was equally visited ($p < 0.05$). Flies were 8–16 days old. Twenty insects were evaluated from 09:00 AM to 13:00 PM per day until a total of 40 insects had been tested. After testing five insects, the entire set-up, *i.e.* all parts of the Y-tube and vials, were rotated 180° to avoid position effects. Data were compared with the Wilcoxon signed-rank test. Data from the first and last insect choices were analyzed by the Chi-square test (SPSS 15.0, SPPSS Inc.).

2.6. Mating assays

Mating trials were conducted in screen cages (30 cm cubes) at 25 °C. By using an aspirator, 25 treated (exposed to oil) and 25 control (non-exposed) males and 25 females were placed into a cage. Trials commenced between 08:00 and 08:30 AM and, mating pairs were collected at 15 min intervals over a 4 h period. Males and females were 7–10 d when tested. Five replicates were done and the assay was repeated twice (n = 10). Oils treatment consisted of exposing males to the vapor phase as follows. A small polypropylene vial containing 20 μL of each essential oil coated with a plastic screen mesh cap to prevent direct contact with the oil source was placed on the bottom of a 3 L glass jar containing 150 mass-reared males. The glass jar was covered with nylon screening. Exposure lasted 1.5 h and was performed the day before the mating trial. After exposure, the essential oil was removed from the glass jar and food and water were added. To ensure that the exact number of males would be available for each assay, a greater number of males were exposed each time. Males were marked with a dot of paint on the thorax, either treated or control males, 2 days before testing, alternating the colors between successive trials. The number of pairs obtained by untreated and treated males was compared with the Student's *t*-test. In order to maintain the same amount of treated and un-treated males in each cage a male from the opposite treatment was removed each time that a mating pair was collected.

2.7. Toxicity by topical application assay on Ceratitis capitata adults

The topical bioassay was done as described previously by López et al. (2011). Randomly selected flies were anesthetized under a stream of nitrogen for a period of 5 min. The immobilized flies were picked up individually and 2 μL of the test solution was applied to the dorsum of each fly by means of an automatic micropipette. The doses used against both sexes were 100, 50 and 10 μg/insect. All doses were prepared from fresh stock solutions obtained by dissolving the oil in acetone. The control experiment was run simultaneously and consisted of the same number of insects treated with 2 μL of acetone. Flies were 3–5 days old when tested. Cypermethrin was included in the assay as a positive control. Doses used for cypermethrin ranged from 0.5 to 25 ng/fly. Mortality was recorded at 24, 48 and 72 h of treatment. Probit analysis was conducted on mortality data recorded after 72 h of treatment to determine the lethal dose for 50% of mortality (LD_{50}) values for each sex. Data were analyzed with the statistical software SPSS 15.0 (SPPSS Inc.).

3. Results and discussion

3.1. Composition of the essential oils

Compounds identification and relative amount composition are presented in Table 1. Thirty-seven compounds were identified in the essential oil from *S. polygama* which amounted 78.1%. Among the major monoterpenes α-pinene (4.2%), sabinene (4.4%), α-terpinene (4.6%) and α-phellandrene (3.3%) were found. Sesquiterpenes were abundant, the main were hydrocarbons such as α-copaene (1.6%), β-caryophyllene (5.1%), (*trans*)-muurola-4(14), 5-diene (4.7%) and δ-cadinene (7.8%). Conversely, the essential oil from *B. spartioides* showed a composition dominated by the monoterpene hydrocarbons α-phellandrene (44.5%), sabinene (20.7%) and β-pinene (15.9%) which together accounted for 91.9% of the total identified (98.9%). Both oils showed α- and β-pinene, camphene, sabinene, myrcene, α-terpinene and α-phellandrene. The only sesquiterpene common to both oils was the β-caryophyllene, but with a trace levels in *B. spartioides* oil.

3.2. Olfactory activity of the oils on C. capitata adults

Essential oils elicited preferences on females and males (Table 2). More than 72% of the total of tested insects moved against

Table 1
Chemical composition of the essential oils from *Schinus polygama* and *Baccharis spartioides*.

RI	Compound	Area (%)	
		S. polygama	*B. spartioides*
815	Hexanal	tr	–
901	n-Nonane	0.5	–
927	Tricyclene	*tr*	*tr*
929	α-Thujene	3.6	–
939	α-Pinene	4.2	1.3
955	Camphene	0.7	1.6
977	Sabinene	4.4	20.7
979	β-Pinene	3.3	15.9
991	Myrcene	2.2	1.2
1008	p-Mentha-1(7),8-diene	–	3.6
1013	Δ-3-Carene	0.4	–
1015	α-Terpinene	4.6	0.6
1023	p-Cymene	–	1.7
1028	α-Phellandrene	3.3	44.5
1029	o-Cymene	1.8	–
1038	(*cis*)-Ocimene	1.7	–
1049	(*trans*)-Ocimene	0.7	–
1062	γ-Terpinene	2.4	–
1087	Terpinolene	–	*tr*
1121	*cis*-2-Menthenol	–	*tr*
1162	Pinocarvone	–	*tr*
1165	Borneol	–	2.2
1177	Terpinen-4-ol		3.9
1187	α-Terpineol	1.9	–
1196	Myrthenal	–	0.6
1207	n-Nonanal	*tr*	–
1386	α-Copaene	1.6	–
1397	β-Elemene	0.7	–
1419	α-Gurjunene	0.7	–
1434	β-Caryophyllene	5.1	*tr*
1498	Bicyclogermacrene	–	*tr*
1438	Aromadendrene	0.6	–
1469	α-Humulene	0.8	–
1485	γ-Muurolene	0.5	–
1492	α-Zingiberene	1.1	–
1495	(*trans*)-Muurola-4(14),5-diene	4.7	–
1512	γ-Cadinene	5.3	–
1514	β-Bisabolene	2.4	–
1529	δ-Cadinene	7.8	–
1545	C15H24 (α-bisabolene tent)	1.5	–
1547	C15H24 (α-cadinene tent)	0.3	–
1582	Spathulenol	–	*tr*
1583	C15H26O	–	1.1
1593	α-4-Copaenol	2.3	–
1611	Isolongifolan-7-alpha-ol	0.8	–
1623	14-Hydroxy-α-muurolene	4.6	–
1669	α-Muurolol	1.6	–
Monoterpene hydrocarbons		33.8	91.1
Oxygenated monoterpenes		1.9	6.7
Sesquiterpene hydrocarbons		33.1	*tr*
Oxygenated sesquiterpenes		9.3	1.1
Total		78.1	98.9
Yield (mL/100 g)		0.4	0.6

Constituents listed in order of increasing retention indices (RI). Temperature-programmed RI referred to n-alkanes, determined on a HP-5MS capillary column. Percentage values less than 0.1% are denoted as *tr* (traces).

Table 2
Responses of males and females of *Ceratitis capitata* in the Y-olfactometer to the essential oils of *Baccharis spartioides* and *Schinus polygama* *vs* a solvent control (acetone).

	Sex	Y-tube arm	Time (s)	First choice[a]	Last choice[a]	χ^2 [b]	p[c]
S. polygama	♀	E. oil	49 ± 9a	14	19	C_f: 0.76	0.384
		Control	56 ± 9a	19	14	C_l: 0.76	0.384
	♂	E. oil	65 ± 12a	13	12	C_f: 3.56	0.059
		Control	27 ± 10a	5	4	C_l: 4.00	0.045
B. spartioides	♀	E. oil	82 ± 9a	23	21	C_f: 8.53	0.003
		Control	27 ± 9b	7	7	C_l: 7.00	0.008
	♂	E. oil	60 ± 10a	16	16	C_f: 0.31	0.577
		Control	47 ± 10a	13	11	C_l: 0.93	0.335

Different letters among treatments in time spent in each arm denotes significant differences at $p = 0.05$ level according to the Wilcoxon signed paired rank test.
[a] Number of insects in the first (C_f) and last choices (C_l) on each arm of the Y-olfactometer.
[b] Chi-square value.
[c] p-Value.

the air stream in the Y-olfactometer, excepting males with the oil of *S. polygama* (45% responded). In the case of the oil from *B. spartioides*, females were significantly attracted by the oil. More than 70% of the total time was spent in the essential oil arm of the Y-olfactometer while males showed no preferences. In addition, records of initial and final choices showed the same trend. In contrast, males appear to prefer the essential oil of *S. polygama* in the Y-olfactometer and females were neither attracted nor repelled by this oil. Statistical analysis showed significant differences for the final choice made by the males.

Among the constituents identified in the oils here presented γ-terpinene, α-pinene and myrcene are recognized as elicitors of electroantennographic recordings of medflies in both sexes (Light et al., 1992). In addition, α-phellandrene, β-pinene, α-terpinene γ-terpinene are monoterpene hydrocarbons with field trapping activity on both sexes (Casaña-Giner et al., 2001). These components were found in both oils except γ-terpinene that was only present in *S. polygma* oil. Interesting enough is that α-copaene, a recognized attractant of medfly males, were found in this oil. Due to olfactory processing of volatile blends has an emergent property leading to behavioral responses in insects (Bruce and Pickett, 2011), the occurrence of these components in the oil of *S. polygama* might be decoded by males, driving them to higher preference over control in the Y-olfactometer test. On the other hand, females were responsive to the oil of *B. spartioides*. It has been reported that p-cymene and borneol attracts more females than males (Casaña-Giner et al., 2001). Both components were found in the oil of *B. spartioides*. Finding mating or oviposition sites is vital for females. Given that p-cymene, borneol, α-phellandrene, α-terpinene, α- and β-pinene and myrcene are components derived from male scent and fruit volatiles (Jang et al., 1989; Light et al., 1992; Casaña-Giner et al., 2001), these monoterpenes in the oil of *B. spartioides* might explain the preferences of females by this oil.

3.3. Mating trials

Prior exposure of males to the essential oils resulted in an increased sexual competitiveness compared to untreated males (Fig. 1). Males exposed to both the essential oil from *S. polygama* as *B. spartioides* obtained statistically higher matings than non-exposed males ($p = 0.0001$ and $p = 0.03$, respectively) with 1.8 and 1.4 as many mating as non-exposed males, respectively. Moreover, the number of matings obtained by males treated with the essential oil from *S. polygama* was statistically higher than the obtained by males treated with *B. spartioides* ($p < 0.05$). Ginger root oil and orange scent exposed males showed similar results (Papadopoulos et al., 2001; Shelly et al., 2004; Papadopoulos et al., 2006). Male medflies exposed to the bark and fruits of guava (*Psidium guava* L.) had a mating advantage over males deprived access to these substrates (Shelly and Villalobos, 2004). Manuka oil is also

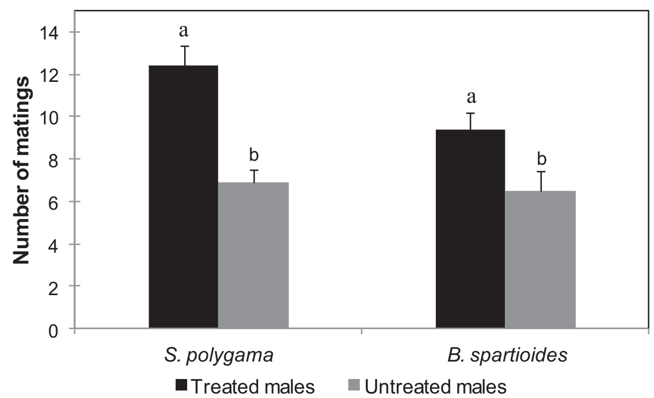

Fig. 1. Enhance on the sexual competitiveness by the essential oils from *Schinus polygama* and *Baccharis spartioides* in pre-exposed (treated) males against non-exposed (untreated) males of *Ceratitis capitata*.

recognized as a powerful attractant to male medflies with similar properties on matings. Additionally, Vera et al. (2013) found that exposure to guava increased the mating success of both wild and laboratory males of *Anastrepha fraterculus* (Tephritidae) relative to non-exposed males and even, the exposed wild males copulated earlier than non-exposed males. All these substances contain the hydrocarbon sesquiterpene α-copaene (Warthen and Mcinnis, 1989; Flath et al., 1994) and, exposure to α-copaene alone was found to increase the mating success of *C. capitata* males (Shelly, 2001). However, some monoterpenes, either acting individually or collectively, can have similar effects on tephritid fruit flies. Recently, Gerofotis et al. (2013) reported that exposure of sexually mature adult olive flies (*Bactrocera oleae*) to the aroma of α-pinene significantly increases the mating performance over non-exposed individuals, both males and female olive flies. Also, Kouloussis et al. (2013) found that a mixture of geraniol,α-pinene, limonene, β-myrcene and linalool increased in a 70% the mating performance of *C. capitata* males.

We performed a complementary study where females were deprived from visual and physical contact to treated and untreated males in the Y-olfactometer. Neither the time spent in each arm nor the initial or final choices were different between female's visits to the scent of males ($p > 0.05$, data not shown). These negative results support the proposed by Shelly et al. (2007a) who postulated that the impact of pre-exposition of males to volatiles on mating success is greatest during close-range courtship.

In this study, irrespective of the compositions of the oils, more females mated with oil treated males. Given that the SIT is an environmentally benign approach for suppressing or eradicating tephritid fruit fly pests and the success of the SIT depends on the ability of released, sterile males to attract, and obtain matings with

Table 3
Probit analysis data for mortality at 72 h after topical application bioassays of different doses of essential oils from *Schinus polygama* and *Baccharis spartioides* on *Ceratitis capitata* adults (N = 30, df: 28).

Essential oil	Sex	LD_{50} μg/insect[95% CL[a]]		Slope ± SE	χ^2
S. polygama	♀	22.0	[17.7–26.9]	3.06 ± 0.26	45.07
	♂	10.3	[8.2–111.5]	5.11 ± 2.58	12.91
B. spartioides	♀	10.7	[6.6–14.8]	1.41 ± 0.19	35.45
	♂	14.6	[8.2–21.2]	1.47 ± 0.19	46.21
Cypermethrin[b]	♀	2.4	[0.9–5.4]	0.76 ± 0.11	73.39
	♂	1.5	[0.7–2.3]	1.06 ± 0.12	37.9

[a] Confidence limits.
[b] Positive control at ng/insect.

wild females, our results have practical importance. The aromatic species tested here proved to be sources of compounds that can modify the sexual performance of sterile medfly males. This is especially important because females display a high degree of mate discrimination, based apparently on male courtship performance (Shelly et al., 2007b). Since the discovery of the enhancement effect of α-copane, Shelly (2001) have promoted the implementation of “aromatherapy” as large-scale exposure of flies to ginger root oil to increase the mating competitiveness of medfly males in sterile release programs. It would be worth now to see whether the enhancement effect found here is higher or last longer than for ginger root oil exposed males.

3.4. Toxic activity of the essential oils

Both oils were toxic for males and females (Table 3). The LD_{50} for the oil of *S. polygama* was 10.3 μg/fly for males and 22.0 μg/fly for females. In contrast, for *B. spartioides* LD_{50} was 10.7 μg/fly for females and 14.6 μg/fly for males, respectively. Essential oils from aromatic species have toxic properties against *C. capitata* adults as those from some *Tagetes* species (López et al., 2011), *Baccharis darwinii* (Kurdelas et al., 2012) and *Azorella cryptantha* (Apiaceae) (López et al., 2012). The essential oils from *B. spartioides* and *S. polygama* showed similar toxic properties to the essential oils from *Tagetes* species and *A. cryptantha* ($DL_{50} \leq 20$ μg/fly).

3.5. Final discussion and conclusion

The aim of this study was to evaluate the semiochemical effect of the essential oils from two native aromatic species as enhancers of sexual competitiveness of sterile males of *C. capitata* as well as potential attractants of adults from both sexes. Shelly et al. (2007a) proposed that the ubiquitous distribution of α-copaene and related compounds among plants, might influence the mating success of *C. capitata* males in the wild. We confirmed by our study in laboratory with the essential oil from *S. polygama*, that native species containing α-copaene and related compounds in the oil account with this potential. Moreover, the essential oils from *B. spartioides* showed a similar effect and, α-copaene or related compounds were absent. All in all, the results might have an impact on SIT programs in two ways: by increasing the efficacy of the SIT if the sterile males increase their competitiveness against wild males and, by increasing the production in mass rearing facilities for the SIT. In addition, lure-and-kill technologies are strategies of chemical control compatible with SIT programs for the control of medfly. Essential oils, such as we evaluated, might be candidate substances for lure-and-kill technologies due to the attractant and toxic properties on medfly adults. Particularly, the fact that *B. spartoides* was highly attractant to females is relevant in the context of finding effective female lures. Further research will include comparison studies with wild populations.

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