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Original Article

The effects of methyl eugenol, cue lure and plant essential oils in rubber foam dispenser for controlling *Bactrocera dorsalis* and *Zeugodacus cucurbitae*

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Abstract

Bactrocera dorsalis and *Zeugodacus cucurbitae* are the most economically important pests of fruits and vegetables production. Male lures like, methyl eugenol (ME) and cue lure (CL) in conjunction with insecticides are commercially used to attract and kill these pests. We investigated, herein, the application of plant essential oils (basil, clove, citronella, and eucalyptus) with ME and CL for both toxicity and attraction. Through the use of rubber foam as a base, basil oil (BO) was found to be highly toxic to *B. dorsalis* and *Z. cucurbitae*, and was evaluated for potency with ME and CL as effective attraction mechanisms. Our results found 15.7-20.3 % of attraction for *B. dorsalis* using ME, separately or mixed with basil oil. The separate CL found 20.3 % while the ME+CL mixture provided 21.5 % attraction for *Z. cucurbitae*. In field tests, a greater number of *B. dorsalis* were trapped with ME alone (20.4 fly/trap/day, FTD), whereas the attraction of the ME and BO mixture was relatively low. For *Z. cucurbitae*, ME+CL (0.1-0.7 FTD) and CL (0.2-0.6 FTD) trapped more flies than mixtures combining BO, suggesting that combinations of BO with ME in rubber foam bases may be a viable alternative for the control *B. dorsalis* but not for *Z. cucurbitae*.

Keywords: Para-pheromone, Mixed lure, Plant essential oils, Rubber foam, Attract and kill, Tephritid fruit fly

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Introduction

The Oriental fruit fly, *Bactrocera dorsalis* (Hendel), and the melon fly, *Zeugodacus cucurbitae* (Coquillett) of the family Tephritidae are the most destructive fruit fly species worldwide, particularly in tropic and sub-tropic regions (Vargas et al., 2000; Prabhakar et al., 2012a; Qin et al., 2018). They cause direct losses to fruit and vegetable crops in the field, as well as indirect losses related to the restrictions on the export of fruits and vegetables due to bio-security concerns of these species in various parts of the world (Aketarawong et al., 2014). These pests have been managed primarily via food bait, bait sprays, para-pheromones, and insecticides for the past century. To control tephritid flies the 'lure and kill' approach, based on food attractants or para-pheromones mixed with a killing agent, have been more recently employed as part of the Integrated Pest Management (IPM) plan (Canale et al., 2013). Due to the increasing demand for organic foods, successful practices of the 'lure and kill' system within the guidelines of an organic farming system are needed.

Male attractants, such as methyl eugenol (ME) and the cue lure (CL) have been successfully used over the past few decades for the detection and control of fruit flies; namely, the oriental fruit fly and melon fruit fly. To date, no fruit fly species have been found to react with combinations of ME and CL, as their responses were deemed to be connected to special antennal receptor sites (Metcalf and Metcalf, 1992). Where such fruit flies prevail, several experiments have been conducted to evaluate the effectiveness of baits mixed with ME and CL for recognition and control of fruit flies. The careful and creative mixing of lures could lessen the number of traps in such areas, as well as reduce the number of laborers necessary to maintain them (Royer and Mayer, 2017).

Excessive use of synthetic pesticides is associated with many environmental problems, such as insecticide resistance, lethality to other organisms, persistence in soil and food, and ecological pollution (Desneux et al., 2007), and are not suitable for organic cultivation. These factors create an urgent need to switch over to natural products that are environmentally friendly, less hazardous to other organisms, and suitable for organic farming. Plant extracts containing insecticidal properties against tephritid fruit flies could be potential alternatives to synthetic insecticides (Benelli et al., 2012), as they are more ecologically friendly than synthetic insecticides (Isman and Machial, 2006). Many plant families contain sufficient amounts of essential oils (EOs) that are toxic to insect pests; such as Apiaceae, Asteraceae, Fabaceae, Lamiaceae, Lauraceae, Poaceae, Myrtaceae, Rutaceae, and Zingiberaceae (Maggi and Benelli, 2018). In our study, we tested four plant essential oils: basil oil; clove oil; citronella oil; and eucalyptus oil, known to possess several volatile bioactive chemicals. Chang et al. (2009, 2013) investigated the insecticidal properties of basil oil (*Ocimum* spp. Lamiaceae) in fruit flies, which produced 99 % adult mortality in *Callosobruchus maculatus* (Fabricius) compared to 0 % for the control. In addition to mortality, basil oil was also found to decrease oviposition and in-egg hatchings (Kéïta et al., 2000). Other broadly used EOs include clove oil (*Eugenia caryophyllata* Spreng, Myrtaceae) and Citronella oil (*Cymbopogon nardus* L. Poaceae). Arancibia et al. (2013) noted the effects of 3 % clove and citronella oils with a soy protein-lignin film incorporation on the time of knockdown of Mediterranean fruit flies (*Ceratitis capitata*) (Wiedemann). Their results showed that vapors emitted from the clove film caused the mortality of 40 % of the flies after four hours of exposure, which increased to 90 % after twenty hours. They determined that the active ingredients of citronella oil may not have released effectively from the film, and, therefore, suggested that citronella oil may be more effective in raw form. In further studies, Ilyas et al. (2017) found that eucalyptus leaf extracts (*Eucalyptus camaldulensis* Dehnh., Myrtaceae) demonstrated satisfactory repellence oviposition inhibition of peach fruit flies; and Rossi and Palacios (2015) determined that LC_{50} of *Eucalyptus cinerea* F.Muell. ex Benth. at 5.5 mg/dm^3 through fumigation assay, killed flies within 15 minutes.

Among tephritid flies, the Mediterranean fruit fly, *C. capitata*, is the most studied species interacting with essential oils (López et al., 2011), whereas *B. dorsalis* and *Z. cucurbitae* investigations with EOs have been less examined (Chang et al., 2009, 2013). Many types of dispensers; such as wood, cardboard, polymer, cotton, lure plug, and wafers have been used with different chemicals for the attraction of fruit flies worldwide. In the present study, we applied a novel approach incorporating rubber foam from natural latex which slowly released small amounts of para-pheromone that can remain active for 90 to 120 days (Malee, 2019).

The goal of the present study, therefore, was to screen plant essential oils with ME and/or CL in rubber foam lures against *B. dorsalis* and *Z. cucurbitae*, to assess the attractiveness of the single and mixed lure methods under both laboratory and field conditions, and to ultimately develop an alternative 'lure and kill' system sustainable in organic farming systems.

Material and Methods

Insect rearing

Insects used in this study were reared from infested guava (*Psidium guajava*) and luffa (*Luffa acutangular)* fruit collected from the agricultural fields of Prince of Songkla University, Hat Yai district, Songkhla province, Thailand (latitude and longitude: 7.004658ºN, 100.503350ºE). The infested fruit with larvae was kept in a transparent plastic box (25×20×15 cm) with a perforated lid for air ventilation, filled with a layer (1 cm) of sterile sawdust (autoclaved and dried) at the bottom of the box as a medium for pupation. Upon the adult fly's emergence, they were transferred to another insect-rearing cage (30×30×30 cm). To maintain the growth of the adult flies, cube sugar, yeast hydrolysate, and water *ad libitum* were provided. Adult *B. dorsalis* and *Z. cucurbitae* were identified from the adult flies ten days post-emergence based on the morphological characteristics described by Prabhakar et al. (2012b) and Drew and Romig (2013).

Pure cultures of *B. dorsalis* and *Z. cucurbitae* were further maintained in separate insect-rearing cages as described above. Laboratory conditions for rearing *B. dorsalis* and *Z. cucurbitae* were maintained at 12:12 hours of light and dark periods at 75-80 % relative humidity (RH) and at temperatures of 27 ± 2 °C. To maintain a continuous stock of experimental flies, adult flies of both sexes of the same species were kept in the same cage for mating and oviposition. Within our experiments, only 10-15 day-old adult flies were used from the stock culture.

Essential oils

Commercial grade of basil (*Ocimum* spp.), clove (*Eugenia* spp.), citronella (*Cymbopogon* spp.), and eucalyptus (*Eucalyptus* spp.) essential oils were purchased from Jiangxi Yisenyuan Plant Species Co., Ltd; China.

Rubber foam preparation

Para rubber foam was prepared using a modification of Dunlop's technique (Calvert, 1982; Malee, 2019). High ammonia concentrated natural latex 60 % (167 g) was first stirred for five minutes to remove the preservative. Other chemical ingredients included potassium-oleate solution 20 % (10 g), Sulphur dispersion 50 % (5 g), zinc-N-diethyldithiocarbamate dispersion 50 % (2 g), zinc-2-mercaptobenzothiazole dispersion 50 % (2 g), butylated hydroxytoluene 50 % (2 g), diphenyl guanidine dispersion 30 % (20 g), zinc oxide dispersion 50 % (20 g), and sodium silicofluoride dispersion 20 % (10 g). Each of the chemicals was added into the latex within ten minutes under continuous stirring. The formed latex was poured into molds and then vulcanized in a hot air oven at 70 ºC for three hours. The resulting para-rubber foam was then dipped into compounded latex in which to produce a thin film over the foam and cut into 2×5 cm pieces for use as dispensers. The para rubber foam dispensers were kept at the ambient temperature before bioassay.

Each plant essential oil, 300 µl of basil oil, clove oil, citronella oil, and eucalyptus oil, was loaded into separate rubber foam dispensers (2×5 cm) using individual 1 ml syringes. The rubber foam dispensers with essential oil, as well as a control (without essential oil) were weathered in a natural environment for 0, 15, 30, and 45 days. Four foam dispensers were selected from each group on each respective day for experimental investigation.

Fumigation toxicity bioassay

In conducting the bioassay, ten *B. dorsalis* and *Z. cucurbitae* adult flies (five male and five female; 10-15 days old) were kept in a clear plastic container (750 ml) with some minute holes in the lid. A rubber foam dispenser with a specified weathering period (0, 15, 30, and 45 days) of essential oil was placed on the bottom of the container to expose the adult flies for a maximum of 72 hours. Yeast, sugar, and water-soaked cotton were provided as food. The experiment was conducted in a Completely Randomized Design (CRD) with four replications for *B. dorsalis* and three replications for *Z. cucurbitae*. Mortality was observed at 1, 6, 24, 48, and 72 hours after exposure. The plant essential oil with the highest percentage of mortality was selected for the next experiment.

The attraction test of the *B. dorsalis* and *Z. cucurbitae* to lures

Methyl eugenol (ME) (4-allyl-1,2-dimethoxybenzene, CAS 93-15-2) and the cue lures (CL) [4-(p-acetoxyphenyl)-2-butanone, CAS 3572-06-3] were supplied by Sigma-Aldrich, St. Louis, MO, USA. One hundred µl of each single ME or CL lure was injected into a rubber foam dispenser. Combinations of the lure (50 µl of ME+CL injected in the same spot and 50 µl of ME/CL injected in a different spot) were injected into a rubber foam dispenser. The pore size of the foam consisted of small holes or pockets in which to retain the liquid chemicals. When we injected both lures (ME and CL) at separate points, they were not mixed but volatilized. Lures injected at the same spot in the dispenser were also volatilized. The experiment followed a Completely Randomized Design (CRD) with five treatments: ME; CL; ME+CL; ME/CL; and the control (foam without chemicals) with four replications of each treatment. After being weathered outside in a natural environment for 0, 15, 30, or 45 days; ten males *B. dorsalis* and ten male *Z. cucurbitae* for each treatment were released in a gauge cage (30×30×30 cm). Yeast, sugar, and water *ad libitum* were supplied. After that, a foam dispenser (previously prepared with 100 µl of the lure) was put above the cage for one hour. The numbers of attracted flies were recorded for each species and calculated as a percentage of attraction.

Attraction test of fruit flies with lures and essential oil at the same point on rubber foam dispensers

Similar amounts of ME, CL, ME+CL, and ME/CL were prepared as described above. Three hundred µl of basil oil was injected in a foam dispenser at the same spot where the lures were previously loaded. For ME/CL, half of the 300 µl of basil oil was separately injected in the same spot as the ME and CL in the rubber foam dispenser. This experiment was also conducted in a Completely Randomized Design (CRD) with five treatments: ME+BO; CL+BO; ME+CL+BO; ME/CL+ BO; and the control with four replications for each treatment. The foam dispensers were then were weathered outside in the natural environment for 0, 15, 30, and 45 days. After that, ten male *B. dorsalis* and ten male *Z. cucurbitae* were released in a gauge cage (30×30×30 cm). The foam dispenser was kept above the cage for one hour. The percentage of attractiveness was calculated for each species within each treatment.

Attraction test of flies with lures and essential oil at different points on rubber foam dispensers

Equal treatment amounts of ME, CL, ME+CL, and ME/CL were prepared as described above. Three hundred µl of basil oil was injected in the foam dispensers at a different point from where the lures were previously loaded. The experiment was conducted in Completely Randomized Design (CRD) for the five treatments; CL/BO, ME/BO, ME+CL/BO, ME/CL/BO, and the control (foam without chemicals) with four replications per treatment. Again, the foam dispensers were weathered in the natural environment for 0, 15, 30, or 45 days; a net cage was prepared, and ten male *B. dorsalis* and ten male *Z. cucurbitae* were released. The foam dispenser was placed above the case for one hour, and the number of flies was recorded for each species for each treatment.

The efficiency of mixed lures and essential oils in field conditions

Field experiments were conducted in the three sapodilla orchards located in Koh Yor, Songkhla province, Thailand: Orchard 1, 7.152179 °N - 100.541570 °W (~0.32 ha); Orchard 2, 7.157172 °N-100.536131 °W (~0.22 ha); and Orchard 3, 7.152701 °N -100.532129 °W (~0.20 ha. The orchards were at distances around 2-3 km apart. Seven selected treatments (ME, CL, ME+CL, ME/BO, CL/BO, ME+CL/BO, and the control underwent three replications each, totaling 21 traps per orchard. The lure (100 µl) and the essential oil (300 µl) were injected in the foam dispenser similar to the previous experiments. The foam lures were then tied into the lids of the clear plastic cup traps (750 ml) with aluminum wire. Four lateral holes, 10 mm in diameter, were made at the upper sidewall of the trap as an entry route for flies. The base portion of the trap container was wrapped with a yellow trap to attract flies.

The experiment design was conducted in Randomized Completely Block Design (RCBD) with seven treatments and three replications (orchards). The traps with only lures (ME, CL, and ME+CL) were prepared with 200 ml of 20 % propylene glycol to attract flies. Propylene glycol was not added into the lures with basil oil (ME/BO, CL/BO, and ME+CL/BO), as an additional killing agent was not needed. The traps were installed under the trees at 150 cm above ground and at a minimum distance of 10 m.

Traps were initially set on 15 Aug 2019, and the first observation began on 1 Sep 2019, which continued at 15-day intervals until the end of the experiment on 2 Feb 2020. Lures were replaced monthly throughout the experiment. Lastly, species were separated and identified in a laboratory setting based on the morphological characteristics under a Leica S8APO stereo zoom microscope (Wetzlar, Germany) according to Drew and Romig, 2013. Meteorological data; such as temperature, relative humidity, and rainfall were also recorded for their various effects upon the experiments' success.

Statistical analysis

Percentages of mortality and attraction were generated under laboratory conditions, transformed by arcsine and square root analyses. Data were subjected to one-way ANOVA and means comparisons were made via Tukey's Honestly Significant Difference Test (α= 0.05). The number of trapped flies within the field conditions was converted to flies per trap per day (FTD), transformed by log (X + 0.5). Repeated-measure ANOVA tests were performed with time as a recurring factor to test for different numbers of FTD between treatments. All analyses were performed using an SPSS 11.0 program for Windows (SPSS, 2001).

Results

Mortality assay for *B. dorsalis* and *Z. cucurbitae*

For *B. dorsalis*, the percentage of cumulative mortality of adult fruit flies varied significantly with each essential oil. Eucalyptus oil outperformed the other oils within 12 hours of exposure, in which 100% mortality was observed ($F_{4,20}$ = 58.412; $p < 0.01$) (Figure 1A). In contrast, other EOs displayed continued activity 24 to 72 hours after exposure. The observations on day 15, after 72 hours, found that basil oil produced 95.0 ± 2.9 % fly mortality, followed by clove oil with 87.5 ± 2.5 % mortality with a significant variation ($F_{4,20}$ = 276.964; $p < 0.001$) (Figure 1B). The other EOs did not show satisfactory effectiveness. On day 30, the basil and clove oils were moderately active with 72.5 ± 2.5 % and 67.5 ± 10.3 %, respectfully, amid further reduced effectiveness from the other essential oils (Figure 1C). Lastly, on day 45, none of the oils remained effective (Figure 1D). And, naturally, no mortality occurred within the control treatment.

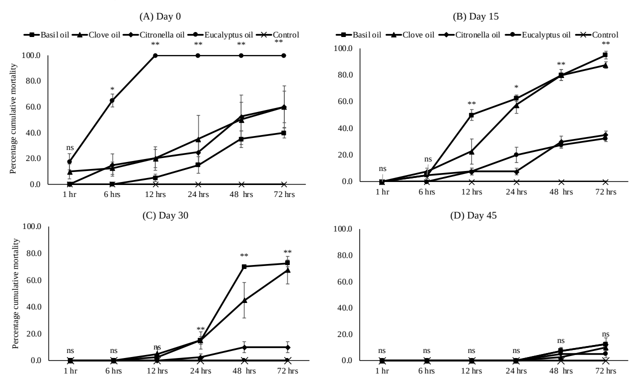

Figure-1. Cumulative mortalities of adult *Bactrocera dorsalis* from rubber foam injected with a plant essential oil (basil oil, clove oil, citronella oil, eucalyptus oil, and the control); weathered for (A) 0, (B) 15, (C) 30, and (D) 45 days.

For *Z. cucurbitae*, the cumulative percentage mortality of adult flies exposed to the essential oils was again significant. Eucalyptus oil again proved superior with 100 % mortality within the first 12 hours of exposure (Figure 2A). By contrast, the other oils were far less effective. On day 15, after 72 hours, basil oil and clove oil produced 76.7 ± 6.7 % and 40.0 ± 11.5 % mortality, respectively ($F_{4,15}$ = 16.562; $p < 0.01$) (Figure 2B). On day 30; basil, clove, and citronella oils were found to produce minimum mortality, yet did not differ significantly (Figure 2C). On the last day of observation, Day 45, none of the oils were effective (Figure 2D). Again, no dead flies were not found in the control.

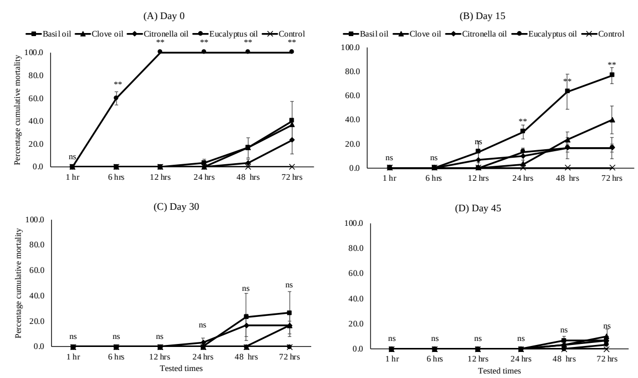

Figure-2. Cumulative mortalities of adult *Zeugodacus cucurbitae* from rubber foam injected with plant essential oils (basil oil, clove oil, citronella oil, eucalyptus oil, and the control), weathered for (A) 0, (B) 15, (C) 30, or (D) 45 days.

The attraction of adult male of *B. dorsalis* and *Z. cucurbitae* to methyl eugenol with a single or mixed cue lure

The percentages of attraction of *B. dorsalis* by the single (ME) and mixed lure (ME+CL and ME/CL) on Day 0 proved significantly different ($F_{4,20}$ = 34.627; $p < 0.001$). The highest percentage of attractiveness was obtained with ME at 32.5 ± 4.8 %. On day 15 through 45, the percentages across all treatments were not significantly different. For *Z. cucurbitae*, the mixed lure (ME+CL) gave higher percentages of attraction on Days 0 and 15 than those of the cue lure (CL). On Days 30 to 45, the percentages of attraction for both the single (CL) and mixed lures (ME+CL and ME/CL) were not significantly different (Table 1).

Attraction of adult male *B. dorsalis* and *Z. cucurbitae* to rubber foam injected with basil oil in the same spot with methyl eugenol and a single cue lure or mixed lure

The percentages of attraction for both *B. dorsalis* and *Z. cucurbitae* decreased when all lures were mixed with basil oil. At Day 0, the percentages of attraction for both species in all treatments ranged from 0.0-8.3 %. For *B. dorsalis*, the percentages of attraction from days 15-45 ranged from 13.3-28.3 %, whereas for *Z. cucurbitae*, the percentages of attraction were less than 15.0 % (Table 2).

Boxplot analyses of the attraction levels were performed from Day 0 to Day 45. The single ME, ME+CL, and ME/CL with and without BO were not significantly different, ranging from 15.5-20.3 %; whereas the single CL and CL with and without BO did not attract this fly species (Figure 3A).

The attraction of adult male *B. dorsalis* and *Z. cucurbitae* to rubber foam injected with basil oil in a different spot with methyl eugenol and a single cue lure or mixed lure

The percentages of attraction for both *B. dorsalis* and *Z. cucurbitae* decreased when all lures were mixed with basil oil at a different spot in the rubber foam. At Day 0, the percentages of attraction for both species in all treatments were less than 20.0 %. For *B. dorsalis*, the percentages of attraction from Days 15-45 were similar to the blended basil oil and lure, which ranged from 10.0-28.3 %. For *Z. cucurbitae*, the percentages of attraction were similar to those of the previous experiment (Table 3).

Boxplot analyses of attraction from Day 0 to Day 45 were performed. The ME+CL (21.5 ± 2.3 %) and CL (20.5 ± 1.5 %) attracted more flies than those mixed with BO (6.7 -13.3 %) (Figure 3B).

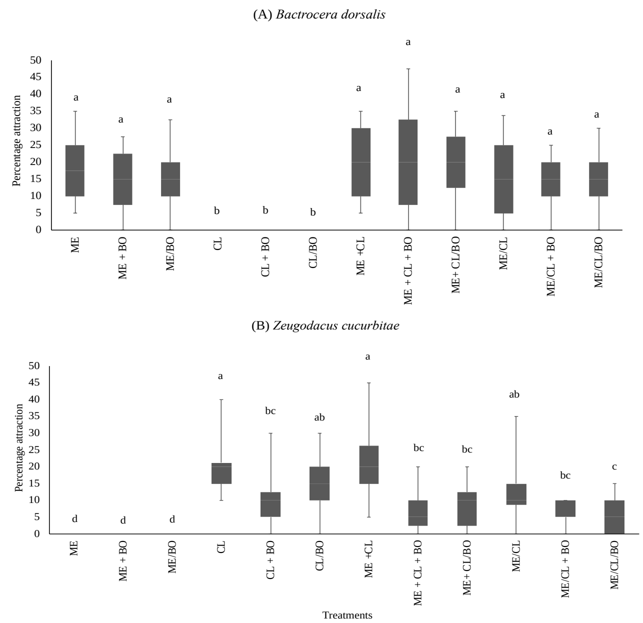

Figure-3. Boxplot analysis of attraction levels of adult *Bactrocera dorsalis* (A) and *Zeugodacus cucurbitae* (B) to rubber foam with methyl eugenol (ME) and single cue lure (CL) or mixed lures with and without basil oil (BO) injected at the same (+BO) or a different spot (/BO).

Table-1. Percentages of attraction of adult male *Bactrocera dosalis* and *Zeugodacus cucurbitae* to methyl eugenol (ME) and a single cue lure (CL) or mixed lure (ME+CL) under laboratory conditions.

Tested day	Fruit fly's species	Mean percentage of attraction (mean)± SE) [1/]				
		ME	CL	ME+CL	ME/CL	Control
0	*B. dorsalis*	32.5 ± 4.8a	0.0 ± 0.0c	22.5 ± 1.4ab	21.3 ± 2.4b	0.0 ± 0.0c
	Z. cucurbitae	0.0 ± 0.0c	17.5 ± 1.4ab	27.5± 4.8a	13.8± 2.4b	0.0± 0.0c
15	*B. dorsalis*	30.0 ± 2.0a	0.0 ± 0.0b	26.3 ± 2.4a	25.0 ± 3.5a	0.0 ± 0.0b
	Z. cucurbitae	0.0± 0.0c	18.8 ± 2.4ab	22.5 ± 3.2a	8.8 ± 3.1b	0.0 ± 0.0c
30	*B. dorsalis*	15.0 ± 2.0a	0.0 ± 0.0b	16.3 ± 2.4a	17.5 ± 1.4a	0.0 ± 0.0b
	Z. cucurbitae	0.0± 0.0b	28.8 ± 4.3a	31.3 ± 4.7a	18.8 ± 6.9a	0.0 ± 0.0b
45	*B. dorsalis*	17.5 ± 4.3a	0.0± 0.0b	18.8± 5.2a	8.8± 3.8a	0.0 ± 0.0b
	Z. cucurbitae	0.0 ± 0.0c	20.0 ± 2.0a	15.0 ± 2.0ab	11.3 ± 2.4b	0.0 ± 0.0c

[1/]Different letters in the same row indicate statistically significant differences by Tukey' HSD test (p<0.01).

Table-2. Percentage of attraction of adult male *Bactrocera dorsalis* and *Zeugodacus cucurbitae* to rubber foam injected with basil oil (BO) in the same spot with methyl eugenol (ME) and a single cue lure (CL) or mixed lure (ME+CL or ME/CL) under laboratory conditions.

Tested day	Fruit fly's species	Mean percentage of attraction (mean) ± SE) [1/]				
		ME+BO	CL+ BO	ME+CL+ BO	ME/CL + BO	Control
0	*B. dorsalis*	5.0 ± 2.9a	0.0 ± 0.0a	1.7 ± 1.7a	3.3 ± 1.7a	0.0 ± 0.0a
	Z. cucurbitae	0.0 ± 0.0a	6.7 ± 1.7a	8.3 ± 4.4a	6.7 ± 3.3a	0.0 ± 0.0a
15	*B. dorsalis*	18.3 ± 7.3a	0.0 ± 0.0b	28.3 ± 7.3a	18.3 ± 6.0a	0.0 ± 0.0b
	Z. cucurbitae	0.0 ± 0.0c	13.3 ± 1.7a	6.7 ± 1.7b	8.3 ± 1.7ab	0.0 ± 0.0c
30	*B. dorsalis*	20.0 ± 7.6a	0.0 ± 0.0b	16.7 ± 6.0a	23.3 ± 4.4a	0.0 ± 0.0b
	Z. cucurbitae	0.0 ± 0.0a	5.0 ± 5.0a	3.3 ± 1.7a	3.3 ± 1.7a	0.0 ± 0.0a
45	*B. dorsalis*	13.3 ± 1.7a	0.0 ± 0.0b	13.3 ± 3.3a	15.0 ± 5.0a	0.0 ± 0.0b
	Z. cucurbitae	0.0 ± 0.0b	15.0 ± 7.6a	6.7 ± 4.4ab	6.7 ± 1.7ab	0.0 ± 0.0b

[1/]Different letters in the same row indicate statistically significant differences by Tukey' HSD test (p<0.01).

Table-3. Percentage of attraction of adult male *Bactrocera dorsalis* and *Zeugodacus cucurbitae* to rubber foam injected with basil oil (BO) in the different spot with methyl eugenol (ME) and a single cue lure (CL) or mixed lure (ME+CL or ME/CL) under laboratory conditions.

Tested day	Fruit fly's species	Mean percentage of attraction (mean) ± SE) [1/]				
		ME/BO	CL/BO	ME+CL/BO	ME/CL/BO	Control
0	*B. dorsalis*	3.3 ± 3.3a	0.0 ± 0.0a	3.3 ± 1.7a	8.3 ± 4.4a	0.0 ± 0.0a
	Z. cucurbitae	0.0 ± 0.0b	18.3 ± 1.7a	3.3 ± 3.3ab	6.7 ± 4.4ab	0.0 ± 0.0b
15	*B. dorsalis*	18.3 ± 10.1ab	0.0 ± 0.0b	23.3 ± 3.3a	13.3 ± 6.0ab	0.0 ± 0.0b
	Z. cucurbitae	0.0 ± 0.0b	15.0 ± 2.9a	6.7 ± 4.4ab	1.7 ± 1.7b	0.0 ± 0.0b
30	*B. dorsalis*	16.7 ± 1.7b	0.0 ± 0.0c	28.3 ± 1.7a	26.7 ± 1.7a	0.0 ± 0.0c
	Z. cucurbitae	0.0 ± 0.0a	6.7 ± 4.4a	6.7 ± 3.3a	5.0 ± 2.9a	0.0 ± 0.0a
45	*B. dorsalis*	15.0 ± 5.0a	0.0 ± 0.0b	15.0 ± 2.9a	10.0 ± 2.9a	0.0 ± 0.0b
	Z. cucurbitae	0.0 ± 0.0b	15.0 ± 2.9a	13.3 ± 3.3a	8.3 ± 1.7a	0.0 ± 0.0b

[1/]Different letters in the same row indicate statistically significant differences by Tukey' HSD test (p<0.01).

Efficiency of the mixed lure and basil oil in field condition

The present study also determined the mean captured fruit flies at fifteen-day intervals for six months. Throughout the trapping period, the ME alone trapped a higher number of *B. dorsalis* than the other treatments, and the trend for trapping was higher during the first three collection dates. The greatest number of *B. dorsalis* captured were in the second collection date (22.5 ± 5.0 FTD) with a highly significant variation ($F_{6,21}$ = 74.124; p < 0.001), followed by the ME/BO trap (14.4 ± 1.4 FTD) (Figure 4A). Upon the fourth collection date, the number of files obtained in each treatment was not significantly different. A fluctuating trend of trapping was observed between the location where ME alone was always higher, followed by either ME/BO or ME+CL/BO with significant variations (Figure 4A).

Conversely, the trapping trend for *Z. cucurbitae* was higher during the first three consecutive collection dates with highly significant variations ($F_{6,21}$ = 4.149; p < 0.001). The ME+CL trap caught the greatest number of *Z. cucurbitae* followed by CL (Figure 4B). At the third collection date (0.8 ± 0.3 FTD) the highest number of flies was found in the ME+CL trap. Afterwards, fluctuations in the trend of trapping by CL and ME+CL were observed. The combination of CL and ME+CL with basil oil showed a reduction in the number of trapped flies in comparison to CL and ME+CL without basil oil, which were just slightly higher than that of the control (Figure 4B).

Boxplot analyses of trapped flies from the 2nd week to the 24th week (six months) were performed. For *B. dorsalis*, ME alone and ME/BO were not significantly different, ranging from 3.8-5.5 FTD; whereas CL alone and CL/BO did not attract *B. dorsalis* (Figure 5A). For *Z. cucurbitae*, CL alone and ME+CL showed the same number of trapped flies with 0.3 ± 0.03 and 0.3 ± 0.06 FTD, respectfully. CL alone and the ME+CL combination with BO presented a decrease in the number of trapped flies with 0.1 ± 0.02 and 0.2 ± 0.05 FTD, respectfully (Figure 5B). *Z. cucurbitae* were not detected in ME, ME/BO, or the control trap. In our field study, we used single lures (ME and CL) and mixed (ME+CL), single lures mixed with basil oil (ME/BO, CL/BO), and mixed lures (ME+CL/BO). We assumed that because basil oil contains ME, the basil oil inclusions would increase the effectiveness of the traps; however, our results found that the most efficient trap was the single ME trap, which was similarly matched by the ME+CL traps. The greatest mean number of flies was achieved with the ME traps, followed by the ME+CL and ME/BO traps. It could be that the ME in basil oil somehow reduced the attraction of ME.

Interestingly, ME+CL outperformed the ME/BO traps, as the mix of the two lures proved more attractive to CL responsive species than those responsive to ME. In the case of *B. dorsalis*, the ME trap was the most attractive. For *Z. cucurbitae*, the ME+CL trap was equally effective as the CL trap, whereas the CL and ME+CL traps with basil oil were less effective than the traps without basil oil. These results suggest that basil oil may inhibit CL activity.

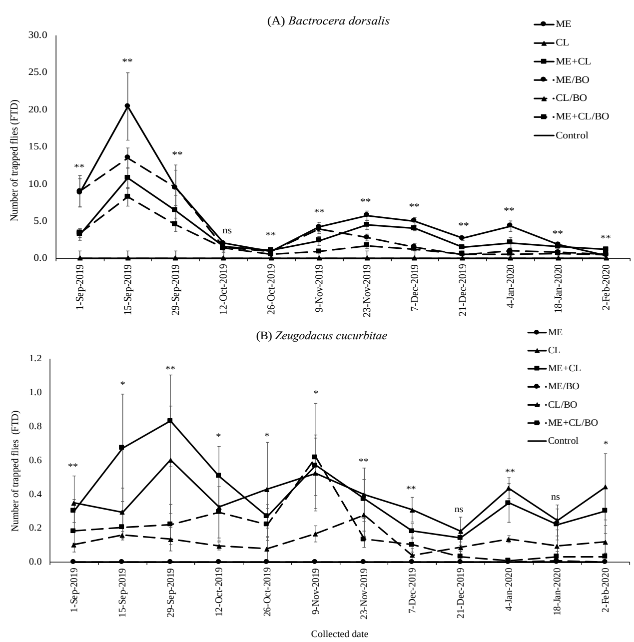

Figure-4. Mean number of trapped flies (FTD) of *Bactrocera dorsalis* (A) and *Zeugodacus cucurbitae* (B) collected every two weeks from the field up to the 24th week (6 months).

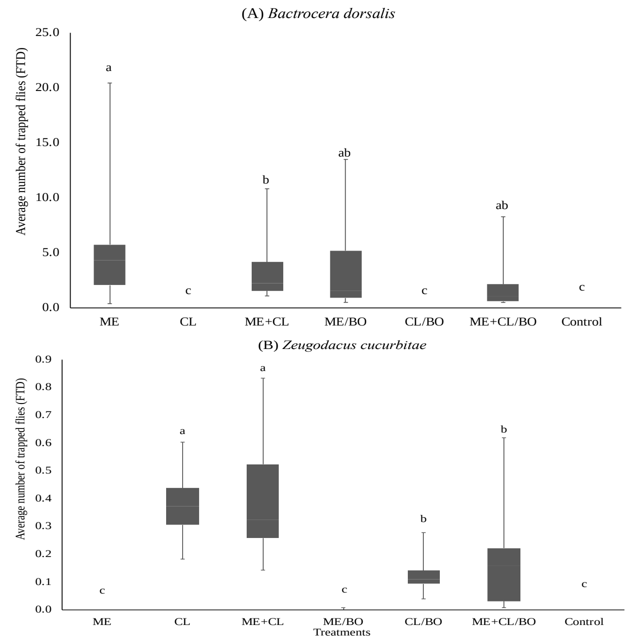

Figure-5. Boxplot analysis of the mean number of trapped flies (FTD) of *Bactrocera dorsalis* (A) and *Zeugodacus cucurbitae* (B) within each lure treatment under field conditions.

Discussion

The insecticidal activity of the essential oils basil, clove, citronella, and eucalyptus were injected in the para-rubber foam against *B. dorsalis* and *Z. cucurbitae* and tested, thereby increasing the number of possible essential oils to combat tephritid fruit flies. The present study evaluated the toxicity of these essential oils, and determined that eucalyptus oil was the most toxic, causing 100 % mortality within 12 hours (Day 0); followed by clove, basil, and citronella oil for both *B. dorsalis* and *Z. cucurbitae*. This general finding is supported by other studies with similar results. Eucalyptus oil proved the most poisonous against the peach fruit fly *B. zonata* (Sanders) pupa (LC_{50} value 38.88 ml/L), followed by basil and other essential oils (Ali, 2018). Hidayat (2014) demonstrated the strong fumigant effects on the first and second instar larvae of the Queensland fruit fly, *B. tryoni* (Froggatt). However, our experiments utilized varied exposure times to the EOs within the rubber foam receptacles and proved no longer useful for both types of flies after extended weathering. The major constituents of eucalyptus oil are monoterpene and sesquiterpene hydrocarbons (Russo et al., 2015). As we have observed, eucalyptus oil was found to be more active in a raw form immediately after exposure.

Basil oil proved the most effective against *B. dorsalis*, more so than *Z. cucurbitae*, after weathering for 30 days. Similar results were found with basil (*Ocimum basilicum* L.) oil, which has three major active structures (trans-anethole, estragole, and linalool) against *B. dorsalis* and *Z. cucurbitae*. The toxic action of basil oil in *C. capitata* occurred significantly faster than in *Z. cucurbitae*, yet moderately faster in *B. dorsalis* (Chang et al., 2009). Among Tephritid fruit flies, the Mediterranean fruit fly is the most investigated species involving interactions with essential oils. When tea tree (*Melaleuca alternifolia*, Myrtaceae) oil was applied in contact and fumigation assay to *C. capitata* and its parasitoid *Psyttalia concolor*, the highly toxic results produced a lower LC_{50} value towards *C. capitata* over its parasitoid *P. concolor* [contact: 0.117 µl oil/cm^2 vs 0.470 µl oil/cm^2; fumigation: 2.239 µl oil/L air vs 9.348 µl oil/L air] (Benelli et al., 2013). In their earlier study, essential oils from fresh rosemary, lavender, chan, poiteau, and arborvitae leaves were imposed upon *C. capitata* and lavender oil proved to be the most toxic at 24 hours (LC_{50} of 9.09 µg/L) (Benelli et al., 2012). Kostyukovsky et al. (2002) determined that some plant essential oils act very quickly and effectively on various insect pests by affecting the neuromodulator octopaminergic. This receptor is absent in mammals, making such actions less hazardous to other non-target organisms and the environment.

Essential clove oil was also found to have a toxic effect on fruit flies of both species. For *B. dorsalis*, death rates were 60.0 % and 87.5 % after 72 hours on Day 0 and Day 15, respectively. For *Z. cucurbitae*, decreased mortality rates of 36.7 to 40.0 % were observed after 72 hours on Days 0 and 15. Further studies in EO toxicity were conducted against Mexican fruit flies *Anastrepha ludens* (Loew). Essential oils from *Eugenia caryophyllus*, *Ocimum basilicum*, and *Thymus vulgaris* were used to determine the ingestion toxicity against adult *A. ludens*; in which *E. caryophyllus* proved the most toxic with LC_{50} 3,529 ppm, followed by *T. vulgaris* (5,347 ppm) and *O. basilicum* (8,050 ppm) (Buentello-Wong et al., 2016). Notably, these EOs were also toxic to *B. dorsalis* and *Z. cucurbitae* under laboratory conditions.

We incorporated basil oil (BO) within the male lures, ME, and CL; at the same and different positions in the rubber foam, and observed their levels of attraction. However, satisfactory results were found only in the single lures. The addition of BO with ME did not increase the effectiveness of ME alone in attracting *B. dorsalis;* and, with CL, a reduction in the attraction of *Z. cucurbitae* was observed. Chang et al. (2009) determined that a mixture of ME and BO did not affect the toxicity of BO to *B. dorsalis*. They surmised the ME may play a physiological role in toxicity reduction. Excessive concentrations of CL (50 and 90 %) in combination with BO decreased the mortality or knockdown in *Z. cucurbitae* males and females. Additionally, a mixture of linalool with CL showed decreased potency to the species as concentrations of CL increased.

Dharmadasa et al. (2015) used two morphotypes of *Ocimum tenuiflorum* (L.) to attract *B. dorsalis* and compared the results with those of ME. Their results focused on the mean number of flies attracted in the first few weeks (53-111 flies), but did not involve mixing plant essential oils with lures. In our experiments with *B. dorsalis*, single ME and ME mixed with BO showed no effect on fly attraction. For *Z. cucurbitae*, the CL and CL mixed with BO showed a relative decrease in attraction. A mix of CL and BO would, therefore, have an antagonistic effect.

Chang et al. (2013) developed a single package of ME+BO with a modified area wide pest management (AWPM) standard trap for *B. dorsalis*. They observed that it took two hours for flies entering the traps to die. Their results also indicated that the ME+BO formulation was effective for 90 days and beyond. They observed differences in the two studies due to the amounts of BO within each dispenser. They found that mortality increased with added amounts in the sponge dispensers (size 7.5 cm x 5 cm) that contained 5,000 µl of ME:BO (1,000 µl: 4,000 µl). In contrast, the rubber foam (size 5 cm x 2 cm) utilized in the study herein contained 400 µl of ME:BO (100 µl: 300 µl). They further observed that only the ME trap caught a greater number of *B. dorsalis* than the ME+BO trap and the control.

Conclusion

Among the four plant essential oils tested, BO proved the most toxic within the first 15-30 days. The single ME and mixture of ME with BO were similar in the attraction of *B. dorsalis*, but for *Z. cucurbitae* the mixture of CL with BO decreased attraction. In the field tests, the single ME performed similarly to the combination of ME and BO in trapping *B. dorsalis*, whereas the combination of CL with BO presented an antagonistic effect in trapping *Z. cucurbitae*. We may, therefore, conclude that a trap with ME and BO may be an effective alternative for the *B. dorsalis* management in organic farming systems, further leading to a reduction of chemical insecticides in the environment.

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Contribution of Authors

Akter MM: Conceived idea, designed research methodology, data collection, data analysis and manuscript writing.
Theary K: Data collection and analysis.
Kalkornsurapranee E: Manuscript final reading and approval.
Prabhakar CS: Designed research methodology, manuscript final reading and approval.
Thaochan N: Conceived and designed the research, manuscript editing, statistical analysis.