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Original Article

The effects of *Metarhizium guizhouense* PSUM02, petroleum oil, and *Azadirachta excelsa* seed kernels extract against *Zeugodacus cucurbitae*

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Abstract

Zeugodacus cucurbitae is a serious agricultural pest of cucurbitaceous crops. *Metarhizium guizhouense* PSUM02, petroleum oil, and *Azadirachta excelsa* seed kernel extract singly and combined were investigated for control of this pest. The angled luffa fruit treated with petroleum oil and *A. excelsa*, or their mixtures showed egg-laying inhibition by 45.3-77.1%. Individually *M. guizhouense* PSUM02 inhibited egg-laying by 25.4%. The mixed application of *M. guizhouense* PSUM02 and *A. excelsa* had negative impacts on the number of eggs and larvae of *Z. cucurbitae*. In field conditions, the average fruit numbers and fruit weights of un-infested angled luffa indicated efficacy similar to malathion for treatment with *M. guizhouense* PSUM02 + petroleum oil and for *M. guizhouense* PSUM02 + petroleum oil + *A. excelsa*. The combined application of *M. guizhouense* PSUM02 with *A. excelsa* or petroleum oil showed negative effects to egg and larval stages of *Z. cucurbitae* providing an alternative strategy for *Z. cucurbitae* control.

Keywords: *Metarhizium guizhouense*, petroleum oil, *Azadirachta excels*, *Zeugodacus cucurbitae*

1. Introduction

The melon fruit fly, *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae) formerly *Bactrocera* (*Zeugodacus*) *cucurbitae*, is an economically important insect pest in the tropical areas including Asia and Southeast Asia (Allwood *et al.*, 1999; Dhillon *et al.*, 2005; Hendrichs *et al.*, 2015), particularly throughout Thailand (Clarke *et al.*, 2001). Their recorded hosts cover more than 81 plant species of the family Cucurbitaceae (Dhillon *et al.*, 2005). The melon fruit flies impact negatively both quality and quantity of fruit (Dhillon *et al*., 2005; Singh *et al*., 2000). Therefore, alternative microbial insecticides and natural products should be investigated for reducing the adverse effects of chemical application.

The management of a wide range of fruit fly pests with entomopathogenic fungi has been intensively studied, for example with *Metarhizium anisopliae* (Mochi *et al*., 2006; Quesada-Moraga *et al*., 2006, 2008; Toledo *et al*., 2007; Yousef *et al*., 2013), and with the species *M. guizhouense* PSUM02 (Thaochan & Chandrapatya 2016; Thaochan & Ngampongsai 2015). Application of *Metarhizium* sp. in fruit crops decreases insect pest population and also reduces crop losses from pest infestation (Ekesi *et al*., 2011). Natural products from plants and petroleum oil have been also reported for controlling this pest. *Azadirachta excelsa* (Jack) Jacobs is suspected to contain biologically active compounds (azadiracthins) that are detrimental to insects (Hummel *et al*., 2012; Schmutterer *et al*., 1993). The seed kernel extracts of this plant contain azadiecthin L (Kalinowski *et al*., 1993; Kanokmedhakul *et al*., 2005), which is effective in the control *Z. cucurbitae* (Muennu *et al*., 2012; Pipithsangchan *et al*., 2006). In addition, petroleum oil was also effective in the control of fruit fly (Nguyen *et al*., 2007; Daniel 2014).

Previous studies have reported that *Metarhizium* sp., pretoleum oil, and *A. excelsa* seed kernel extracts are compatible for combined use, and this enhanced efficiency of the insect pathogenic fungus (synergistically or additively) for the control of insect pests (Haroon *et al*., 2011; Loongsai *et*

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al., 2012; Shah *et al.,* 2008). In the current study, we investigated the effects of *M. guizhouense* PSUM02, petroleum oil, and *A. excelsa* seed kernel extract, individually and in mixtures, on egg-laying, and on immature and adult stage development of *Z. cucurbitae,* under laboratory and greenhouse test conditions. Moreover, select treatments were further applied to suppress *Z. cucurbitae* infestation in an angled luffa crop, in field test conditions. The control of *Z. cucurbitae* with these products might contribute to the successful management of this pest in cucurbitaceous crops, and could reduce pesticide use.

2. Materials and Methods

2.1 Insect collection and culture

Infested angled luffa fruit (*Luffa acutangula* (L.) Rox) with fruit fly larvae were collected from an orchard in Hat Yai district, Songkhla province, Thailand, and were kept in clear plastic boxes (20 × 25 × 15 cm) with perforations on the lid for ventilation. The bottom of each box was covered with a 1 cm layer of sterile sawdust for pupation. The pupae were sieved and kept in a clear plastic box (10 × 10 × 10 cm). After eclosion, adult fruit flies were transferred to a gauze cage (30 × 30 × 30 cm) and reared with cube sugar, water and yeast hydrolysate. *Zeugodacus cucurbitae* were identified when they were 10 days old based on the morphological characters described by Hardy (1973), White and Elson-Harris (1992), and Drew and Hancock (1994).

After identification, the *Z. cucurbitae* were reared in a cage (30 × 30 × 30 cm) and provided with cube sugar, a water soaked sponge cloth, and yeast hydrolysate, which were changed weekly. The flies were maintained in the insect rearing room with natural photoperiod (12:12 h of light:dark), natural relative humidity (75-80%) and ambient temperature (28 ± 2°C). The male and female flies were kept together in the same cage until they mated. The female flies were reproductively mature at 15–20 days age.

2.2 Fungal strain

Metarhizium guizhouense PSUM02 was obtained from the culture collection at the Natural Biological Control Research Center (NBCRC), Southern Region, Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University (Thaochan & Chandrapatya, 2016). Slant monoconidial cultures of the strain were grown on Sabouraud dextrose agar plus yeast extract (SDAY) (10 g/L dextrose, 2.5 g/L peptone, 2.5 g/L yeast extract, and 20 g/L agar) for 15 days at 27 ± 2°C in darkness. The viability of conidia was assessed by spreading 500 µl of 1×10^6 conidia/ml suspension on SDAY, and incubating at 27 ± 2°C in complete darkness for 24 hours. The percentage germination was determined by assessing 100 conidia at 400× magnification. Conidia with germ tubes longer than their width were considered viable, and the viability was higher than 95%.

2.3 A. excelsa seed kernel extracts

The seeds of *A. excelsa* were collected in Hat Yai district, Songkhla province, Thailand. Ten kilograms of fresh seed kernels of *A. excelsa* were blended and transferred into a 20 L glass bottle, and 15 L of methanol was added as a solvent. The fresh seed kernels were incubated for seven days for maceration and extraction. The extracted sample was filtered through Whatman #1®, and the solvent removed in a rotary evaporator. The extract was incubated at 60°C for 60-180 min to remove solvent remnants. This was repeated for seven batches of fresh seed kernels. The extraction products were kept in a refrigerator at 4°C until use.

2.4 Effects of *M. guizhouense* PSUM02, petroleum oil and *A. excelsa* seed kernel extract, individually and in mixtures, on egg-laying inhibition of *Z. cucurbitae*

Un-infested angled luffa fruit (indicated by the absence of scars or scratches) were collected from the experimental field of Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University. Eighteen angled luffa fruit that were 10 days old after fruit setting were collected, washed with tap water for 5 min, and dried with paper towels. The fruit were then cut to 10 cm length and divided into two equal halves that serve as dome-like structures for collecting the eggs, with totally 36 domes. The cut luffa fruit with dome shape was suitable for collecting and observing the eggs in the luffa fruit. Each dome was repeatedly pierced with an entomological pin (number 3) to make 40-50 tiny about evenly distributed holes on the surface of each. Four fruit domes were sprayed with 0.5 ml of each single treatment (Table 1) and let dry at room temperature for 1 h. Petroleum oil (SK99®, 83.9% W/V EC) (Sotus International Co., Ltd., Thailand) and malathion (Eramol 83, 83% W/V EC) (Erawan Agricultural Chemical Co., Ltd., Thailand) were purchased from the market. Malathion and water were set as the positive and the negative control, respectively. Malathion is an organophosphate (OP) insecticide that is a neurotoxin (Environmental Protection Agency, 2012).

Each treated fruit dome was placed alone in a 12 cm diameter plastic dish lined with a black-colored Whatman #1® 9.0 cm filter paper. Before the domes were placed in the dishes, they were sprayed with water to simulate the surface of a fruit, in order to facilitate oviposition. One fruit dome of each single treatment was exposed for oviposition, for 24 h, in a holding gauze cage (30 × 30 × 30 cm) containing 10 gravid 20 day-old female *Z. cucurbitae*, with totally four cages per treatment. The flies were used only once in an experiment and then discarded. These eggs were carefully assessed with a stereo microscope and counted. The number of eggs from each treatment was converted to the percentage of egg-laying inhibition following the equation (Sabatini *et al.,* 2001):

$$\text{Egg laying inhibition (\%)} = \frac{[\text{No. Egg in control} - \text{No. Egg in treatment}]\times 100}{\text{No. Egg in control}}$$

Table 1. Individual and mixture treatments with *Metarhizium guizhouense* PSUM02, petroleum oil, and *Azadirachta excelsa* seed kernel extract.

	Treatment	Concentration
1	*Metarhizium guizhouense* PSUM02 (M)	1×10^8 spore/ml
2	Petroleum oil (SK99®) (P)	2,000 ppm
3	*A. excelsa* seed kernel extract (A)	100,000 ppm
4	M + P	
5	M + A	
6	P + A	
7	M + P +A	
8	Malathion	1,500 ppm
9	Water	-

Petroleum oil, *A. excelsa* seed kernel extract and Malathion were mixed with water to make a final concentration.

2.5 Effects of *M. guizhouense* PSUM02, petroleum oil, and *A. excelsa* seed kernel extract, individually and in mixtures, on the immature stage development of *Z. cucurbitae* in laboratory and greenhouse bioassays

2.5.1 Laboratory bioassays

Thirty-six un-infested 10 day-old angled luffa fruit, sampled after fruit setting from an experimental field, were washed with tap water for 5 min and dried with towel paper. Four fruit were sprayed (0.5 ml) with each treatment (Table 1) and let dry for 1 h at room temperature. Then one fruit of each single treatment was exposed for oviposition, for 24 h, in a holding cage (30 × 30 × 30 cm) containing 10 gravid 20 day-old female *Z. cucurbitae* with totally four cages per treatment. This experiment used entire fresh fruit for larval growth and development. The flies were used only once in an experiment and then discarded. The infested fruit were placed in their individual clear plastic boxes (20 × 25 × 15 cm) with perforations on the lid for ventilation. The bottom of each box was covered with a one-centimeter layer of sterile sawdust for pupation. The larvae, pupae, and adult stage *Z. cucurbitae* from each infested angled luffa fruit were counted. The larvae were counted when the 3rd instar larvae were released into the sawdust by opening the infested fruit.

2.5.2 Greenhouse bioassays

The angled luffa plants were planted in plastic pots (40 × 30 cm), two plants per pot. A total of 64 pots were transferred, placing 16 pots to each of the four greenhouse cages (2 × 2 × 2.5 m) at an experimental field of the Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University. This experiment was done in April – July, 2013 (rainfall 4.4 ± 2.4 mm, temperature 28.0 ± 0.3, and 78.4 ± 1.6 %RH). The angled luffa fruit were tested at 10 days of age, after fruit setting. In each greenhouse cage, six angled luffa fruit were randomly sprayed 1 ml with each of the six treatments in Table 1 (1, 4, 5, 7, 8 and 9), so a total of 36 fruit per cage were treated. The fruit were dried in greenhouse cage conditions for 24 h. Four hundred 20 day-old gravid female flies were transferred into the greenhouse cages, 100 flies per cage, and allowed to lay eggs over 24 and 48 h. Three fruit of each treatment at 24 and 48 h were collected and moved to clear plastic boxes in the laboratory. The 20.0 cm × 25.0 cm × 15.0 cm boxes had perforated lids for ventilation. The bottom of each box was covered with a one-centimeter layer of sterile sawdust for pupation. The larvae, pupae and adult stage *Z. cucurbitae* from each treatment were counted.

2.6 Application of *M. guizhouense* PSUM02, petroleum oil and *A. excelsa* seed kernel extract to angled luffa crop under field conditions

Three experimental fields were used: two of them at the Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University; and one in Khlong Hoi Khong district, Songkhla province, Thailand. This experiment was done in January – April, 2014 (rain fall 1.0 ± 1.3 mm, temperature 27.4 ± 1.2 and 76.8 ± 2.2 %RH). Each 400 m^2 field was divided to 100 m^2 plots. The angled luffa plants spanned six rows with 14 plants per row in a 100 m^2 plot, so each field had 336 plants (four plots, six rows each, 14 plants each). Four select treatments from Table 1 (4, 7, 8 and 9) were used in each field, so each plot was assigned a treatment. The angled luffa fruit were first sprayed 40 days after planting or 5 days after fruiting. Then the treatments were sprayed with knapsack sprayer providing 5 L/100 m^2 every seven days, until 82 days after planting. Plastic screens were used to protect against contamination between treatments during spraying. The fruit were collected starting 50 days after planting, or 10 days after fruiting, and then every two days over 32 days, for 16 collection dates total. Total fruit weights and counts of infested and un-infested fruit were recorded.

2.6.1 Data analysis

All parameters of egg-laying inhibition and immature stage counts in laboratory conditions were analyzed. Then for each parameter of egg-laying inhibition, and immature stage counts in both laboratory and greenhouse conditions, were compared between the various treatments by the analysis of variance (one-way ANOVA), in a completely randomized design and randomized complete block design with four replications of each treatment. The fruit weights and counts in field conditions were analyzed by one-way ANOVA similar to the greenhouse test, with three replications. The Tukey's Honestly Significant Difference Test ($\alpha = 0.05$) was used to compare means of the experimental treatments. All statistical analyses were carried out with the SPSS 17.0 program for Windows (SPSS 2008) (Windows EDU S/N 5065845).

3. Results

3.1 Egg-laying inhibition

The average inhibition of egg-laying by gravid female *Z. cucurbitae*, in angled luffa in the laboratory, was affected by the treatment ($F = 114.77$; df = 8, 27; $P < 0.01$) (Figure 1). Treatment with water served as the negative control with zero inhibition of egg-laying, while Malathion served as the positive control and had the highest 96.2 ± 2.8% average inhibition. Most of the flies that contacted Malathion

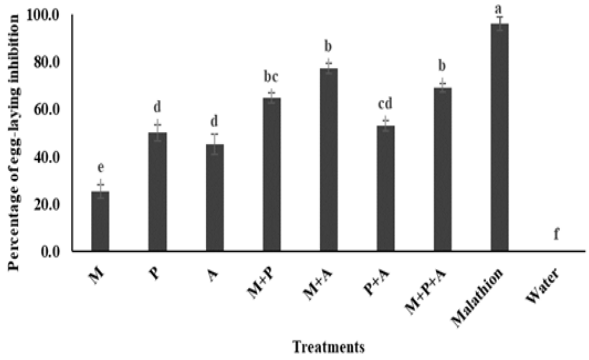

Figure 1. The egg-laying inhibitions (mean ± SEM) of *Metarhizium guizhouense* PSUM02 (M), petroleum oil (P) and *Azadirachta excelsa* seed kernel extract (A) (Table 1) applied to angled luffa against gravid female *Zeugodacus cucurbitae* (Couqillett) in a laboratory test. Malathion was the inhibiting control, and water was the non-inhibiting control. Different letters indicate statistically significant differences at the $P < 0.01$ level, according to Tukey's HSD test. N = four replications; one fruit dome per replication

treated angled luffa died before laying eggs. When applied by itself, *M. guizhouense* PSUM02 gave a low 25.4 ± 3.0% average egg-laying inhibition, while with petroleum oil and *A. excelsa* seed kernel extract the resulting 45.3 – 50.1% inhibition was significantly better. The treatments with mixtures gave mutually similar average inhibition in the range 53.1 – 77.1%.

3.2 Immature stage development and adult Emergence laboratory conditions

The average counts of immature and adult stages of *Z. cucurbitae*, in the variously treated angled luffa in laboratory conditions, are summarized in Table 2. Treatment with water gave the highest 165 ± 8.9 count of larvae that significantly differed from the other treatments ($F = 42.86$; df = 8, 27; $P < 0.01$). Among the single (non-mixed) treatments, *M. guizhouense* PSUM02 had the second highest 126 ± 6.0 larvae/fruit, and was followed by petroleum oil and *A. excelsa* seed kernel extract in this order. The average larval counts with mixture treatments ranged within 32-76 larvae/fruit. Malathion gave the lowest 9 ± 5.8 larvae/fruit, significantly differing from the other treatments.

The average counts of pupae were similar to those of larvae (Table 2). The water treatment gave the highest 121 ± 10.5 average number of pupae. Across all treatments (including those with mixtures) the pupae counts ranged within 22-69. Malathion gave the lowest 2 ± 1.5 pupae/fruit, and significantly differed from the other treatments ($F = 43.24$; df = 8, 27; $P<0.01$). Treatment with *M. guizhouense* PSUM02 showed the highest average 39 ± 3.3 count of unemerged pupae that significantly differed from the other treatments ($F = 66.19$; df = 8, 27; $P<0.01$). For the other treatments, the average number of unemerged pupae was in the range 0-9.

The average number of adult emergence of *Z. cucurbitae* from angled luffa was the highest at 113 ± 10.1 flies/fruit when treated with water, and this was significantly different from the other treatments ($F = 44.69$; df = 8, 27; $P<0.01$). Treatment with *M. guizhouense* PSUM02 gave on average 13 ± 0.9 flies/fruit, and this was not significantly different from Malathion with 2 ± 1.5 flies/fruit. All the other treatments gave from 11.3 to 60.5 flies/fruit.

3.3 Greenhouse conditions

The five best treatments were selected based on the laboratory bioassays, giving the strongest negative effects on immature stage development and adult emergence of *Z. cucurbitae.* These treatments were further studied in the greenhouse conditions. Water and Malathion were used as the control treatments. At 24 h after treatment, each tested treatment had significant effects on larvae ($F = 10.48$; df = 5, 18; $P<0.01$), pupae ($F = 10.02$; df = 5, 18; $P<0.01$), unemerged pupae ($F = 4.92$; df = 5, 18; $P<0.01$) and adults ($F = 12.99$; df = 5, 18; $P<0.01$) (Table 3). The angled luffa treated with water showed the highest average count of 60 ± 6.1 larvae, followed by *M. guizhouense* PSUM02 with 58 ± 4.5 larvae. The other treatments with mixtures ranged from 24 to 47 larvae/fruit. Malathion gave the lowest count of 13 ± 1.7 larvae/fruit (Table 3).

Table 2. Counts (mean ± SEM) of immature stages and adult emergence of *Zeugodacus cucurbitae* in a laboratory test with the treatments in Table 1.

Treatment	Larvae	Pupae	Un-emerged pupae	Adults
M	126 ± 6.0b	52 ± 3.0bc	39 ± 3.3a	13 ± 0.9de
P	106 ± 13.7bc	68 ± 8.9b	8 ± 1.4b	61 ± 8.5b
A	81 ± 6.1cd	38 ± 4.7cd	0 ± 0.0c	38 ± 4.7bc
M+P	76 ± 7.0cd	26 ± 0.8de	3 ± 1.2bc	23 ± 1.4cde
M+A	32 ± 4.6ef	31 ± 2.3cd	4 ± 0.7bc	27 ± 1.9cd
P+A	62 ± 5.1de	53 ± 3.3bc	8 ± 1.8b	45 ± 4.3bc
M+P+A	39 ± 6.1ef	20 ± 3.0de	9 ± 1.1b	11 ± 2.8de
Malathion	9 ± 5.8f	2 ± 1.5e	0 ± 0.0c	2 ± 1.5e
Water	165 ± 8.9a	121 ± 10.5a	9 ± 0.6b	113 ± 10.1a

The treatment labels are M = *Metarhizium guizhouense* PSUM02; P = Petroleum oil; A = *A. excelsa* seed kernel extract. Different letters within one column indicate significant differences ($P < 0.01$) using Tukey's HSD test. N = four replications; one fruit per replication

Table 3. Counts (mean ± SEM) of immature stages and adult emergence of *Zeugodacus cucurbitae* for select treatments from Table 1, in a greenhouse test at 24 h.

Treatment	Larvae	Pupae	Un-emerged pupae	Adults
M	58 ± 4.5a	53 ± 5.0ab	14 ± 2.3a	39 ± 4.5ab
M+P	24 ± 3.0bc	21 ± 3.1cd	4 ± 1.3b	18 ± 1.8cd
M+A	47 ± 10.9ab	43 ± 10.3abc	6 ± 4.6ab	37 ± 7.0abc
M+P+A	29 ± 5.1bc	28 ± 4.7bcd	1 ± 0.7b	27 ± 4.2bcd
Malathion	13 ± 1.7c	12 ± 1.7d	1 ± 0.6b	11 ± 1.1d
Water	60 ± 6.1a	58 ± 5.9a	1 ± 0.5b	57 ± 5.8a

The treatment labels are M = *Metarhizium guizhouense* PSUM02; P = Petroleum oil; A = *A. excelsa* seed kernel extract. Different letters within one column indicate significant differences ($P < 0.01$) using Tukey's HSD test. N = four replications; three fruit per replication

The average numbers of pupae were similar to those of the larvae. Treatment with water gave the most 58 ± 5.9 pupae/fruit, followed by the treatment with *M. guizhouense* PSUM02 at 53 ± 5.0 pupae/fruit. The mixtures gave 21-43 pupae/fruit and did not significantly differ from Malathion, which gave the lowest 12 ± 1.7 pupae/fruit. The number of unemerged pupae was the highest at 14 ± 2.3 pupae/fruit when the angled luffa were treated with *M. guizhouense* PSUM02, and this was significantly different from the other treatments ($F = 4.92$; df = 5, 18; $P<0.01$) (Table 3).

In the counts of adult emergence, the treatments with *M. guizhouense* PSUM02 + petroleum oil (M+P; 18 ± 1.8 flies/fruit) or with *M. guizhouense* PSUM02 + petroleum oil + *A. excelsa* seed kernel extract (M+P+A; 27 ± 4.2 flies/fruit) were comparable to malathion (11 ± 1.1 flies/fruit) but significantly different from water (57 ± 5.8 flies/fruit) ($F = 12.99$; df = 5, 18; $P<0.01$) (Table 3).

The treatment labels are M = *Metarhizium guizhouense* PSUM02; P = Petroleum oil; A = *A. excelsa* seed kernel extract. Different letters within one column indicate significant differences ($P < 0.01$) using Tukey's HSD test. N = four replications; one fruit per replication

At 48 h after treatment, in terms of the larval counts the treatments with *M. guizhouense* PSUM02 + petroleum oil (M+P; 26 ± 1.0 larvae/fruit) and with malathion (33 ± 3.0 larvae/fruit) were comparable and significantly different from water (56 ± 4.4 larvae/fruit) ($F = 5.24$; df = 5, 18; $P<0.01$) (Table 4). The average number of pupae with *M. guizhouense* PSUM02 and all the mixture treatments ranged within 25-46 pupae/fruit, and these were not significantly different from Malathion (31 ± 3.2 pupae/fruit), but differed significantly from water (52 ± 3.8 pupae/fruit) ($F = 4.33$; df = 5, 18; $P<0.01$). The average number of unemerged pupae was the highest 16 ± 2.1 pupae/fruit with *M. guizhouense* PSUM02, and this was significantly different from the other treatments ($F = 18.68$; df = 5, 18; $P<0.01$) (Table 4).

The average number of adult emergence was in the range 25-39 flies/fruit with *M. guizhouense* PSUM02 and all the mixture treatments, and these were comparable to Malathion (28 ± 2.4 flies/fruit) but significantly different from water (51 ± 3.6 flies/fruit) ($F = 4.53$; df = 5, 18; $P<0.01$) (Table 4).

3.3 Field test conditions

The two most effective treatments from the greenhouse testing, namely M+P and M+P+A, were chosen for further field tests, along with water and Malathion as the control treatments. The angled luffa fruit weights and counts are summarized in Table 5, and these are shown at various collection times in Figures 2 and 3.

The average total fruit weight and total fruit count were in the ranges 75.8-126.8 kg and 564-811 fruit across all treatments, and showed no significant differences between the treatments. The average un-infested fruit weights with the treatments M+P (101.9 ± 16.4 kg), M+P+A (82.1 ± 20.5 kg), and malathion (94.0 ± 5.5 kg) were comparable, but significantly different from water treatment (38.8 ± 4.4 kg) ($F = 4.64$; df = 3, 8; $P<0.05$).

In the average number of un-infested fruit, the treatments M+P and M+P+A were similar. Their counts of un-infested fruit were not significantly different from Malathion (604 ± 25.5 fruit), but were significantly different from water (329 ± 15.3 fruit) ($F = 11.32$; df = 3, 8; $P<0.05$) (Table 5).

The ranges of infested fruit weight and infested fruit count were 20.0-30.3 kg and 172-235 fruit across all treatments, and showed no significant differences between the treatments.

4. Discussion

The aim of a mixture of different control agents is either to achieve higher efficiencies or to increase reliability. The *M. guizhouense* PSUM02 applied together with *A. excelsa* seed kernel extract or petroleum oil may act independently and be directed at different targets in the insect pest; their effects could be simply additive. However, they may also complementarily improve sensitivity of the target organism and ideally interact synergistically. On the other hand, other competitive interactions are possible leading to antagonistic effects. Our results on the use of *M. guizhouense* PSUM02, petroleum oil, *A. excelsa* seed kernel extract, used singly or as mixtures to treat angled luffa fruit, showed additive effects by suppression of the egg-laying behavior, the immature stages development, and the adult emergence of melon fruit fly *Z. cucurbitae*, when compared to water as control treatment. The treatments with petroleum oil and *A. excelsa* seed kernel extract, when used singly, inhibited egg-laying by 45.3-51.35%, whereas *M. guizhouense* PSUM02 treatment had the lowest 25.4% inhibition (Figure 1). Treatments with mixtures increased the egg-laying inhibition to 53.1-77.1%. The *M. guizhouense* PSUM02 mixed with petroleum oil or *A. excelsa* seed kernel extract or both showed higher efficiency of egg-laying inhibition than the components when used singly.

Table 4. Counts (mean ± SEM) of immature stage and adult emergence of *Bactrocera cucurbitae* for various select treatments in a greenhouse test at 48 h.

Treatment	Larvae	Pupae	Un-immerged pupae	Adults
M	49 ± 3.1ab	46 ± 3.1ab	16 ± 2.1a	31 ± 2.5c
M+P	26 ± 1.0c	25 ± 0.9b	0 ± 0.0c	25 ± 0.9c
M+A	40 ± 7.5abc	39 ± 7.2ab	1 ± 0.4c	38 ± 7.4ab
M+P+A	48 ± 6.9ab	46 ± 7.4ab	8 ± 2.6b	39 ± 6.1ab
Malathion	33 ± 3.0bc	31 ± 3.2ab	3 ± 0.9bc	28 ± 2.4c
Water	56 ± 4.4a	52 ± 3.8a	1 ± 0.3c	51 ± 3.6a

The treatment labels are M = *Metarhizium guizhouense* PSUM02; P = Petroleum oil; A = *A. excelsa* seed kernel extract. Different letters within one column indicate significant differences ($P < 0.01$) using Tukey's HSD test. N = four replications; three fruit per replication

Table 5. The fruit weight (kg) and the fruit number (mean ± SEM) of angled luffa in a field test of select treatments.

Treatment	Fruit weight (kg)			Fruit number (fruit)		
	Total	Un-infested	Infested	Total	Un-infested	Infested
M+P	126.8 ± 26.7	101.9 ± 16.4a	24.9 ± 10.9	811 ± 102.9	629 ± 23.4a	182 ± 79.9
M+P+A	110.7 ± 33.1	82.1 ± 20.5ab	25.0 ± 10.6	735 ± 135.6	523 ± 71.4a	212 ± 74.1
Malathion	114.0 ± 10.5	94.0 ± 5.5ab	20.0 ± 6.9	776 ± 72.0	604 ± 25.5a	172 ± 61.6
Water	75.8 ± 11.8	38.8 ± 4.4c	30.3 ± 9.6	564 ± 93.0	329 ± 15.3b	235 ± 86.7

The treatment labels are M = *Metarhizium guizhouense* PSUM02; P = Petroleum oil; A = *A. excelsa* seed kernel extract. Different letters within one column indicate significant differences ($P < 0.05$) using Tukey's HSD test. N = three replications; 84 plants per replication

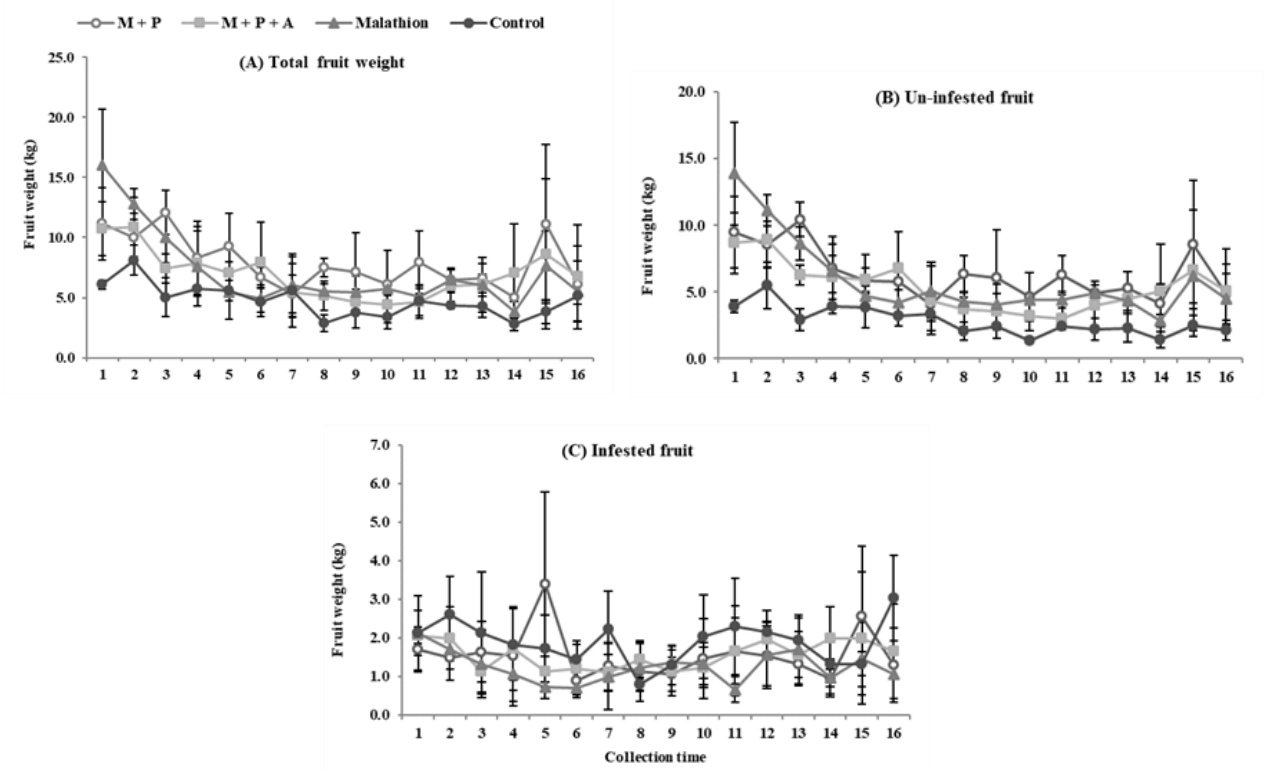

Figure 2. Effects of the treatments with *Metarhizium guizhouense* PSUM02 (M), petroleum oil (P) and *Azadirachta excelsa* seed kernel extract (A) against *Zeugodacus cucurbitae* (Couqillett) infestation, on the fruit weight (kg) of angled luffa (mean ± SEM) (A = total, B = un-infested and C = infested) in a field test. Malathion and water were set as the positive and the negative control treatments. The fruit were collected starting 40 days after planting, or 5 days after fruiting, and then every 2 days over 32 days, for 16 collection dates total.

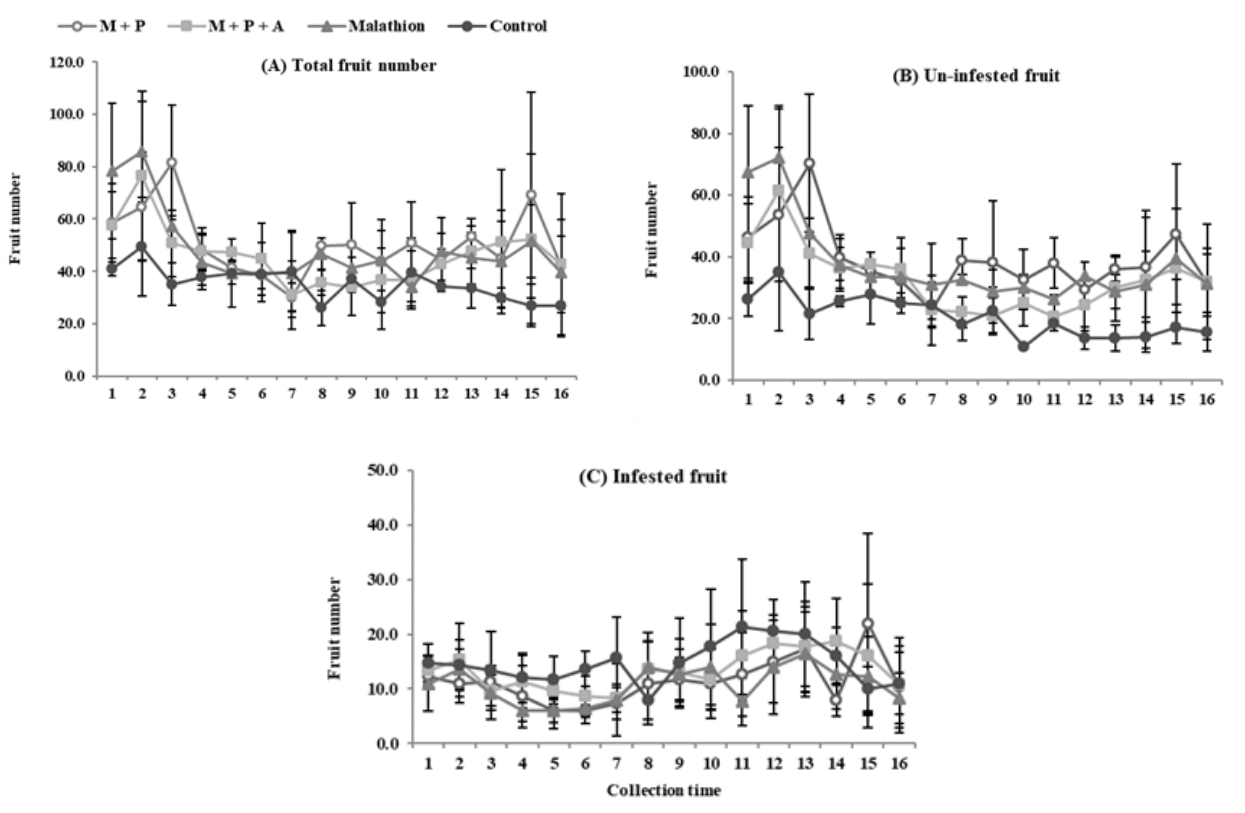

Figure 3. Effects of the treatments with *Metarhizium guizhouense* PSUM02 (M), petroleum oil (P) and *Azadirachta excelsa* seed kernel extract (A) against *Zeugodacus cucurbitae* (Couqillett) infestation on number of angled luffa fruit (mean ± SEM) (A = total, B = un-infested and C = infested) in a field test. Malathion and water were set as the positive and the negative control treatments. The fruit were collected starting 40 days after planting, or 5 days after fruit setting, every 2 days over 32 days, for a total of 16 collection dates.

Singh and Singh (1998) reported that neem seed kernel extract of *A. indica* (1.25-5.0%) significantly deterred oviposition of both *B. dorsalis* and *Z. cucurbitae*. Also, the extract of *A. indica* at the concentration 1-3% showed on average 90.1% deterred oviposition of *Z. tau* (Walker) (Thakur & Gupta, 1998). Prior published research on the effects of petroleum oil and *A. excelsa* seed kernel extract, on repellency and anti-oviposition behavior of tephritid fly support our new results as reasonable (Daniel, 2014; Muennu *et al.*, 2006; Pipithsang chan *et al.*, 2006).

For the immature stage development and adult emergence, both in the laboratory and in the greenhouse test, the treatments with *A. excelsa* seed kernel extract showed low counts of each stage of the fruit fly (Tables 2, 3 and 4). The mixture treatments decreased larval, pupal, and adult emergence counts. *A. excelsa* seed kernel extract restrained the gravid female flies from laying their eggs in the treated host fruit, and this gave low numbers of offspring in each stage of the fruit fly (Ali *et al.*, 2011; Pipithsangchan *et al.*, 2006; Silva *et al.*, 2012). The mixed application of *M. guizhouense* PSUM02 with *A. excelsa* seed kernel extract or petroleum oil may enhance efficiency of the entomopathogen. Otieno *et al*. (2016) observed that combined use of Neem Azal-T (1% azadirachtin) with entomopathogenic fungi (*M. anisopliae* 2539 IPP) and entomopathogenic nematode (*Steinernema carpocapsae*) gave the most efficient killing of the target pest with 65% reduction of adult emergence. Akbar *et al*. (2005) also hypothesized that the prolonged intermoult period of insect larvae by growth-regulating action of azadirachtin may give time for the establishment and penetration of fungal conidia through the insect's cuticle. Evidence for the additive or synergistic interactions of neem and entomopathogenic fungi against armyworms, *Spodoptera litura* (Fabricius), and *Bemisia tabasi* (Gennadius), has been shown by Mohan *et al*. (2007) and Islam *et al*. (2011).

Interestingly, the angled luffa treated with *M. guizhouense* PSUM02 alone, both in the laboratory and in the greenhouse test, showed the highest number of un-emerged pupae. After the different control agents were sprayed singly or mixed on angled luffa fruit, the conidia of *M. guizhouense* PSUM02 may have adhered on the fruit surfaces. Thaochan and Benarlee (2014) reported that the conidia of *M. anisopliae* PSUM04 could survive and adhere on plant tank surfaces for more than one month. During the gravid female laying their eggs on the surfaces of sprayed host fruit, the ovipositor and eggs may become contaminated with *M. guizhouense* conidia. The conidia have the opportunity for adherence and penetration through the integument of the egg or the larval stage. This phase corresponds to adherence and penetration starting with conidia in contact with the cuticle, through germination and the presence or absence of differentiation into apressoria, which, according to Roberts *et al.* (1991), are initial events in the mechanism of infection by entomopathogenic fungi. Other studies have documented adverse

effects of *M. anisopliae* on larvae, pre-pupae, pupae and emergent adults, increasing the mortality of the fly at each stage (Beris *et al.*, 2013; Destéfano *et al.*, 2005).

On the other hand, Depieri *et al.* (2005) and Rachappa *et al.* (2007) reported the inhibition of entomopathogenic fungi by azadirachtin. High concentration of the botanical insecticides used to control insects and diseases have negative effects on vegetative growth and spore production of *M. anisopliae* (Niassy *et al.*, 2012). Some neem-based products in concentrations of 5% a.i. or greater also negatively affected the vegetative growth and conidiogenesis of *B. bassiana* spores (Castiglioni *et al.*, 2003). Amutha *et al.* (2010) reported that 3% azadirachtin was slightly harmful to *B. bassiana*. For *M. guizhoense* PSUM02, the *A. excelsa* seed kernel extract showed less negative effects on vegetative growth and spore production (Loongsai *et al*., 2012).

In the field test, the treatments with M + P or M + P + A were not significantly different from Malathion, in the average fruit weight or in the count of un-infested angled luffa fruit (Table 5). These treatments gave similar negative impacts on *Z. cucurbitae* infestation in the field test. In prior research the mixed application of *Metarhizium* sp. or other entomopathogenic fungi with neem seed extract enhanced the efficiency by up to 10% relative to treatment with the fungus alone, in controlling insect pests (Haroon *et al.*, 2011; Shah *et al.*, 2008).

We have demonstrated that, in laboratory, greenhouse, and field test conditions, the mixed application of *M. guizhouense* PSUM02 with petroleum oil or *A. excelsa* seed kernel extract negatively affects the egg laying by gravid female *Z. cucurbitae*, and also adversely affects the immature stage development and adult emergence. These mixture treatments were more efficient than the fungus alone, in controlling the insect and decreasing the number of insect pests infesting the host fruit (Akbar *et al*., 2005; Islam *et al*., 2011; Mohan *et al*., 2007; Otieno *et al*., 2016). These mixture treatments could replace the use of synthetic insecticides, and are considered safer. The treatments studied are particularly attractive for the control of insect pests in such situations where synthetic insecticides are not permitted.

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