

Full Length Research Paper

Preparation and use of oil formulations of *Beauveria bassiana* and *Metarhizium anisopliae* against *Spodoptera litura* larvae

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In vitro, the compatibility of eight vegetable oils, used as components in formulation, was studied in conidia of entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, based in three parameters that are been evaluated: germination rate, vegetative growth and conidiogenesis. Almond oil and gingelly oils at 1, 2 and 3% concentrations showed compatibility with *B. bassiana* as well as *M. anisopliae*. Mustard oil and eucalyptus oils at all three concentrations proved toxic to *B. bassiana* and *M. anisopliae* except at 1% concentration. Sunflower oil, olive oil, coconut oil and castor oils displayed compatibility with *M. anisopliae* and toxic to *B. bassiana* except olive oil and castor oil at 1% concentration. Conidiogenesis appear to be more affected than germination for the sample which displayed toxicity. Compatibility classification in to toxic, moderately toxic and compatible enabled assessment of the tested oils for use in formulations. Conidial formulations of *B. bassiana* and *M. anisopliae* with almond oil/olive oil/gingelly oil/castor oil were used for bioassaying against *Spodoptera litura*. All the four formulations displayed higher mortalities of the target pest compared to unformulated conidia.

Key words: Entomopathogenic fungi, compatibility, vegetable oils, concentrations, bioassay.

INTRODUCTION

The cutworm *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae), is a major destructive polyphagous pest of subtropical and tropical crops, causing serious economical losses (Rao et al., 1993). Concerns about the negative effects of chemical insecticides have led to emphasis on alternative strategies for pest control. The demand for organically grown food warrants methods that utilize non-chemical inputs for pest control to reduce harmful side-effects of pesticides on public health and environment (Hazra et al., 1998). Pest management

involving biocontrol agents is assuming prominence and have been considered as an important strategy in insect population reduction. *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin and *Paecilomyces fumosoroseus* (Wize) Brown & Smith were recognized as important entomopathogens (Wanida and Poonsuk, 2012; Shoab et al., 2012; Meikle et al., 2005).

In the field, however, the higher temperature, lower humidity and exposure to ultraviolet (UV) radiation could

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Table 1. Isolates of entomopathogenic fungi obtained from USDA/ARSEF collection used in the study of compatibility.

Isolate	Acc. No. ¹	Fungal species	Insect host	Order	Geographical origin
M20	ARSEF1823	<i>M. anisopliae</i>	<i>Nilaparvata lugens</i>	Homoptera	India
M48	ARSEF 1882	<i>M. anisopliae</i>	<i>Tibraca limbativentres</i>	Hemoptera	Brazil
B55	ARSEF 654	<i>B. bassiana</i>	<i>Nilaparvata lugens</i>	Homoptera	China
B51	ARSEF 502	<i>B. bassiana</i>	<i>Ostrinia nubilalis</i>	Lepidoptera	China

¹Accession numbers of strains obtained from the ARSEF-USDA.

be detrimental to the conidia. The situation warrants shift to use of oil-formulation of fungi which showed good results in biological control of insect pests under field conditions (Bateman et al., 1993; Feng et al., 1994; Batta, 2003) Formulations of *M. anisopliae* and *B. bassiana* have been used against *Sitophilus oryzae*, *Rhizopertha dominica* (Batta, 2008); *Tribolium confusum* Duval (Michalaki et al., 2006); *Anopheles gambiae* and *Anopheles stephensi* (Bukhari et.al., 2011) as biocontrol agents.

Studies of cotton seed oil formulations with *Metarhizium flavoviride* against *Schistocerca gregaria* showed increased of infectivity to insects, even at low humidity and high temperatures (Bateman et al., 1993). Furthermore, oil affords protection to fungal conidia from the UV of sunlight (Moore et al., 1993). A number of vegetable oils have been used at different concentrations for preparing conidial formulations of entomopathogenic fungi. Fifty percent oil formulations of *B. bassiana* (Luz and Batagin, 2005); 10% coconut oil formulations (Perry et al., 2005) and 20% oil formulations of *M. anisopliae* against ticks (Hedimbi et al., 2011); 19% coconut oil and 28% soybean oil formulations of *B. bassiana* against almond bark beetles (Batta, 2007) 50% rapeseed and camella oil formulations of *Zoophthora radicans* against *Plutella xylostella* (Batta et al., 2011).

In the present study, compatibility of eight vegetable oils with *B. bassiana* and *M. anisopliae* conidiospores was studied at three concentrations using germination, vegetative growth and conidiogenesis as parameters. Selected formulations were tested against *S. litura* larvae for understanding efficacy of the formulated conidia.

MATERIALS AND METHODS

Fungal cultures

The strains of *M. anisopliae* and *B. bassiana* were obtained from ARSEF-USDA collection (Agricultural Research Service Collection of Entomopathogenic Fungi - United States Department of Agriculture) (Table 1). The monosporic cultures were maintained on Sabouraud's dextrose yeast extract agar (SDAY) stored at 4°C and subculture of the fungal strains was done at two month intervals. The spores from 14-day-old cultures were used for preparing formulations.

Preparation of formulations

Oil-in-water formulation was prepared by mixing the surfactant

mixed oil phase with the spore suspension in aqueous phase. *M. anisopliae* strains were cultured on SDAY for 14 days at 25 ± 2°C, spores were harvested using 0.01% Tween-80 and spore suspensions were prepared by centrifuging the conidia in 0.02% Tween-80 and decanting the supernatant in the centrifuge tubes. The suspension was thoroughly mixed using a vortex mixer, after adding sterile distilled water followed by centrifugation and decantation. The procedure of washing the conidia was repeated three times to eliminate Tween-80. The washed conidia suspended in distilled water, formed the conidial stock 200 µl which was mixed with 9.8 ml of distilled water. Required concentration of conidia was prepared using Neubauer haemocytometer. Oil phase of the conidial samples were prepared with sterilized almond oil, olive oil, sunflower oil, gingelly oil, coconut oil, castor oil, mustard oil and eucalyptus oil at three concentrations (1, 2 and 3%). Triton X - 100 was used as nonionic surfactant, Na₂CO₃ (Sodium Carbonate) as stabilizer and Silicon as antifoaming agent. One percent oil formulation consisted of 1% oil, 1% Triton X - 100, 0.5% silicone, 1% Na₂CO₃ and 96.5% of the aqueous phase. For 2% and 3% formulations the concentration of oil as well as surfactants was increased to twice and thrice respectively. The mixtures of these two phases were then homogenized using the magnetic stirrer for 60 minutes, to get a stable formulation.

Germination assessment

Fifty micro liters of the oil formulation at 3 × 10⁶ conidia/ml was used for inoculating SDAY plates by spread plate method, and four sterile cover slips were randomly placed on each plate. Plates were sealed with parafilm and incubated at 25±1°C. After 24 h post incubation, 1 ml of formaldehyde (0.5%) was transferred onto each plate to arrest germination as per the method of David et al. (2008). Each cover slip was removed and placed on glass slide for making germinated/un-germinated spore count (500 per each cover slip). For each sample three replicates were observed.

Vegetative growth and conidiation

By using the cork borer, a hole with 5mm was made in the middle of SDAY plate and inoculated with 50µl of the formulation at 3 × 10⁶ conidia ml⁻¹ and the plates were sealed with parafilm before incubating at 25 ± 1°C. Colony diameter was recorded on 14th day. For assessment of conidiogenesis, the spores were flushed out from the plates using 10ml of 0.02% Tween-80. Spore count was done using Neubauer haemocytometer and three replicates were maintained for each sample. Data was submitted to ANOVA and means were computed by the Tukey test (p ≤ 0.05).

Compatibility assessment of the different oils was made using the formula Alves et al. (1998)

$$T = \frac{20(VG) + 80(SP)}{100}$$

Where, vegetative growth (VG) and sporulation (SP) were given in

Table 2. Effect of 2% oil formulation on conidia of *B. bassiana* and *M. anisopliae*.

Oil name	Colony diameter ¹		Conidia number/plate ¹	
	Mean(cm)	Reduction percentage	Mean	Reduction percentage
a). Conidia of <i>B. bassiana</i>				
Almond oil	4.27 ± 0.25 ^{c(2)}	15.78	21.8 × 10 ⁸ ± 7.23 × 10 ^{4b}	20.04
Olive oil	4.27 ± 0.32 ^c	15.78	12.7 × 10 ⁸ ± 7.00 × 10 ^{3c}	53.38
Sunflower oil	4.13 ± 0.27 ^d	18.41	12.8 × 10 ⁸ ± 3.46 × 10 ^{3c}	52.98
Gingelly oil	4.43 ± 0.22 ^b	12.49	18.8 × 10 ⁸ ± 3.79 × 10 ^{4b}	31.09
Coconut oil	4.03 ± 0.33 ^d	20.38	11.5 × 10 ⁸ ± 5.86 × 10 ^{3d}	57.95
Castor oil	4.63 ± 0.23 ^b	8.54	15.5 × 10 ⁸ ± 3.06 × 10 ^{4c}	43.33
Mustard oil	4.17 ± 0.47 ^d	17.75	10.2 × 10 ⁸ ± 1.15 × 10 ^{3d}	62.45
Eucalyptus oil	4.03 ± 0.27 ^d	20.38	9.70 × 10 ⁸ ± 4.73 × 10 ^{3d}	64.49
Control I	4.83 ± 0.43 ^a	4.59	27.7 × 10 ⁸ ± 5.20 × 10 ^{4a}	-1.28
Control II	5.07 ± 0.23 ^a	0	27.3 × 10 ⁸ ± 10.0 × 10 ^{4a}	0
b). Conidia of <i>M. anisopliae</i>				
Almond oil	4.87 ± 0.27 ^c	16.57	7.4 × 10 ⁸ ± 1.67 × 10 ^{3d}	26.55
Olive oil	4.8 ± 0.30 ^c	17.71	8.8 × 10 ⁸ ± 2.91 × 10 ^{4b}	12.66
Sunflower oil	5.03 ± 0.29 ^b	13.71	6.05 × 10 ⁸ ± 2.91 × 10 ^{4e}	39.92
Gingelly oil	5.23 ± 0.42 ^b	10.29	9.7 × 10 ⁸ ± 3.45 × 10 ^{4a}	3.72
Coconut oil	4.7 ± 0.22 ^c	19.43	8.02 × 10 ⁸ ± 4.91 × 10 ^{4c}	20.35
Castor oil	5.03 ± 0.49 ^b	13.71	9.37 × 10 ⁸ ± 2.91 × 10 ^{4a}	6.95
Mustard oil	4.27 ± 0.22 ^d	26.86	5.57 × 10 ⁸ ± 4.36 × 10 ^{3e}	44.67
Eucalyptus oil	4.03 ± 0.29 ^d	30.86	6.05 × 10 ⁸ ± 1.86 × 10 ^{3e}	39.95
Control I*	5.43 ± 0.57 ^a	6.86	9.5 × 10 ⁸ ± 1.86 × 10 ^{4a}	5.71
Control II**	5.83 ± 0.39 ^a	0	10.7 × 10 ⁸ ± 2.60 × 10 ^{4a}	0

¹ Mean of three replicates; ² Means followed by the same letter on column are not significantly different by Tukey test ($p \leq 0.05$); *additives without oil; **without oil and additives.

relation to the control (100%). T value of 0 to 30 = very toxic; 31 to 45 = toxic; 46 to 60 = moderately toxic; > 60 = compatible.

Efficacy test of formulations by laboratory bioassay against *S. litura* larvae

Four oil formulations were prepared using gingelly oil, castor oil, almond oil and olive oil with each of the two strains of *B. bassiana* and *M. anisopliae*. *S. litura* larvae were treated as a batch of 20 kept in perforated plastic boxes by spray application of 2% oil formulation at 10⁸ conidia/ml using automiser. Fresh castor leaves were provided as feed every day and containers were cleaned of insect litter daily. They were placed in an environmental chamber set at 25±1°C. The insects were treated for two consecutive days and controls were treated with an equal volume of water with 0.02% Tween 80®. Bioassays were set up with three replicates for each treatment. Mortality data was collected at 24 h intervals. The dead insects were transferred to moist chambers done by Petri dishes autoclaved with a moist filter paper to facilitate mycosis. Before transferring the dead insects into the chambers, their surfaces were immediately sterilized with 1% sodium hypochlorite followed by three rinses with sterile distilled water. The bioassays were repeated twice. The median lethal time (LT₅₀) was calculated from the cumulative mortality data on each day post treatment, using probit analysis (Finney, 1971) and SPSS-11.

RESULTS AND DISCUSSION

There was a variable reduction in spore germination, in vegetative growth and in conidia production in the different formulations of oil to *B. bassiana* and *M. anisopliae*. Compatibility levels differed significantly between the formulations of the two entomopathogens. Formulations of almond oil and Gingelly at 1, 2 and 3% were compatible with *B. bassiana* and to *M. anisopliae* (Table 2). Mustard oil and eucalyptus oil at three concentrations were classified as toxic to *B. bassiana* and *Metarhizium anisopliae*, except in the concentration of 1% for the fungus *M. anisopliae*. On the other hand, Sunflower oil, olive oil, coconut oil and castor oil formulations showed compatibility with *M. anisopliae* and toxicity with *B. bassiana*. Where as mustard oil and eucalyptus oil showed moderately toxic effects on *B. bassiana* at 1% oil concentration and toxic at 2 and 3% concentrations. On the other hand, with *M. anisopliae*, 1% concentration of oils was compatible while at 2 and 3% moderate toxicity was observed. The antifungal activity of Eucalyptus oil was attributed to its active ingredient citronellal. The antifungal activity of citronellal,

Table 3. "T" values and compatibility classification of eight oils on *B. bassiana* and *M. anisopliae*.

Oil name	T Value					
	1% oil concentration		2% oil concentration		3% oil concentration	
	<i>B. bassiana</i>	<i>M. anisopliae</i>	<i>B. bassiana</i>	<i>M. anisopliae</i>	<i>B. bassiana</i>	<i>M. anisopliae</i>
Almond oil	88.55(C) ²	80.81(C)	79.96 (C)	73.45(C)	71.25(C)	63.28(C)
Olive oil	68.97(C)	92.72(C)	46.62(MT)	87.34(C)	43.61(T)	77.58(C)
Sunflower oil	56.64(MT)	63.36(C)	47.02(MT)	60.05(C)	40.04(T)	50.79(MT)
Gingelly oil	74.15(C)	97.44(C)	68.91(C)	96.28(C)	62.12(C)	78.41(C)
Coconut oil	52.07(MT)	87.76(C)	42.05(T)	79.65(C)	39.40(T)	72.70(C)
Castor oil	64.95(C)	94.38(C)	56.67(MT)	93.05(C)	50.18(MT)	75.02(C)
Mustard oil	46.35(MT)	61.87(C)	37.55(T)	55.33(MT)	35.60(T)	48.47(MT)
Eucalyptus oil	44.91(T)	65.59(C)	35.51(T)	60.05(MT)	31.15(T)	51.61(M)
Control I ¹	101.64(C)	94.38(C)	101.28(C)	94.29(C)	99.18(C)	92.06(C)
Control II ²	100.00	100.00	100.00	100.00	100.00	100.00

C = compatible, MT = moderately toxic, T = toxic, ¹Control I = additives without oil, ²Control II = without oil and additives.

against several species of *Aspergillus*, *Penicillium* and *Eurotium* (Nakahara et al., 2003), and, that mustard oil were reported (Nielsen and Rios, 2000; Dhingra et al., 2004).

Barring few exceptions, all the formulations at the three concentrations and with *B. bassiana* as well as with *M. anisopliae*, germination recorded more reduction rather than the corresponding values for colony diameter. Number of conidia produced showed correspondence to the vegetative growth of the colony measured in terms of colony diameter rather than to percentage of germination. Except that of almond oil the four formulations showed marked reduction in conidiogenesis compared to germination and vegetative growth in *B. bassiana*. With respect to *M. anisopliae* Sunflower oil, eucalyptus oil and mustard oils showed more reduction in conidiogenesis than germination and vegetative growth. It is evident that non-compatible oils show their adverse effect mainly on conidial production. Hence conidiogenesis can be taken into consideration for deciding compatibility of the given oil. Because in case of *B. bassiana* Almond oil is the only oil which is more compatible (Table 3), and it showed less reduction in conidiogenesis compared to germination. Almond, olive, gingelly, coconut and castor oils displayed high compatibility to *M. anisopliae* and also showed less reduction in conidiogenesis, compared to germination except gingelly and castor oils at 3% concentration (Figure 1).

Except mustard oil, the rest of the oils used contain unsaturated fatty acids such as linoleic acid and oleic acids in different proportions, which have anti-fungal properties. Linoleic acid reduced mycelial growth of *Rhizoctonia solani*, *Pythium ultimum* and led to a significant reduction in growth of *Crinipellis perniciosa* (Walters et al., 2004). Coconut oil contains 90% saturated fatty acids, and of these, lauric acid accounts for 45-48% inhibition of spore germination and radial

growth of *Aspergillus niger* (Řiháková et al., 2002). Sunflower oil contains lecithin, tocopherols and carotenoids, but according to Muley et al. (2009) essential oils that contain carotenoids showed anti fungal activity. Drastic reduction in germination and conidiogenesis of *B. bassiana* in the formulation with sunflower oil and coconut oil in the present study may be due to antifungal activity of the components. Qualitative and quantitative composition of fatty acid components of vegetable oils, and surfactants as well as of the insect epicuticle were shown to affect development of *B. bassiana* against *Triatoma infestans* (Luz and Batagni, 2005).

The four oil formulations (Almond, olive, gingelly and castor oils) tested against 3rd instar larvae of *S. litura* displayed high mortality values of 94.5 to 98.6 compared to that of unformulated sample (Table 4) and profuse mycosis and sporulation was observed on the dead cadavers (Figure 2). *Metarhizium* strains M20 and M48 showed maximum mortality values of the larvae treated with gingelly oil formulations. On the other hand *Beauveria* strains B51 and B55 demonstrated maximum mortality in the treatment with Almond oil formulation. However LT₅₀ values were least (4.58-4.89 days) both in *M. anisopliae* and *B. bassiana* strains in castor oil formulation, compared to that of unformulated sample (6.72 - 7.01 days). The Relative Virulence Indices calculated based on the three parameters; viz: LT₅₀, percentage mortality and percentage mycosis were maximum with castor oil for *Metarhizium* strains and with Almond oil treatment for *Beauveria* strains.

Oil in water formulations of *Isaria tenuipes* and *Nomuraea rileyi* caused high mortality levels against *Spodoptera* spp. (Vega-Aquino et al., 2010). Results of the present experiment clears that oil in water formulation at low concentration (2% oil in water) increases efficacy against 3rd instar larvae of *S. litura* compared to *B.*

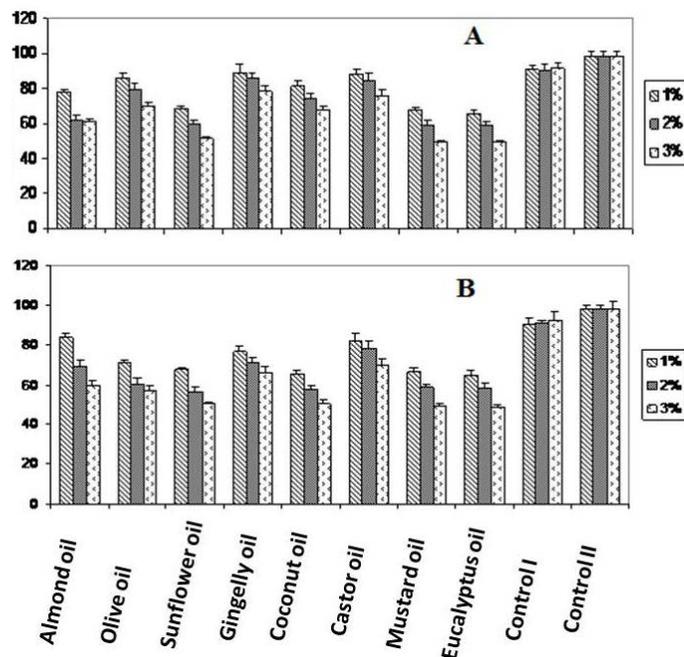


Figure 1. Percentage of conidial germination along with different concentrations (1, 2 and 3%) of vegetable oils after 24 h of incubation, A) *Metarhizium anisopliae*; B) *Beauveria bassiana*.

Table 4. Effect of *Beauveria bassiana* and *Metarhizium anisopliae* oil formulations on 3rd instar larvae of *Spodoptera litura*.

Isolate	Formulation type	LT ₅₀ (in days)	Mortality%	Mycosis%	RVI*
<i>M. anisopliae</i> ARSEF 1823 (M20)	Almond oil	5.11	91.5 ± 0.26 ^{ab (1)}	94.3 ± 0.67 ^{ab}	0.12
	Olive oil	5.66	90.3 ± 1.02 ^{ab}	95.2 ± 0.19 ^{ab}	-0.07
	Gingelly oil	4.66	98.6 ± 0.52 ^b	96.5 ± 0.19 ^b	0.75
	Castor oil	4.58	97.3 ± 0.38 ^b	96.0 ± 0.68 ^b	0.84
	Unformulated	6.72	85.3 ± 0.21 ^a	75.3 ± 0.41 ^a	-1.31
<i>M. anisopliae</i> ARSEF 1882 (M48)	Almond oil	6.14	90.3 ± 0.58 ^{ab}	92.5 ± 0.43 ^b	0.06
	Olive oil	6.39	90.2 ± 0.12 ^{ab}	93.9 ± 0.67 ^b	0.01
	Gingelly oil	5.99	96.7 ± 0.43 ^b	94.3 ± 0.69 ^b	0.65
	Castor oil	4.82	95.2 ± 0.72 ^b	94.1 ± 1.41 ^b	0.92
	Unformulated	7.01	85.1 ± 0.38 ^a	59.1 ± 1.90 ^a	-1.41
<i>B. bassiana</i> ARSEF 654 (B55)	Almond oil	4.69	94.3 ± 0.13 ^b	97.5 ± 0.93 ^b	0.88
	Olive oil	5.63	89.4 ± 0.89 ^{ab}	70.3 ± 1.90 ^c	-1.61
	Gingelly oil	5.08	93.8 ± 1.01 ^b	95.5 ± 0.49 ^b	0.34
	Castor oil	4.69	92.5 ± 0.64 ^{ab}	72.5 ± 1.61 ^c	-1.40
	unformulated	6.73	85.4 ± 0.75 ^a	92.3 ± 0.21 ^{ab}	-1.67
<i>B. bassiana</i> ARSEF 1725 (B51)	Almond oil	4.95	93.1 ± 0.31 ^{ab}	95.5 ± 0.89 ^b	0.83
	Olive oil	5.72	88.2 ± 0.59 ^a	75.1 ± 1.38 ^c	-1.26
	Gingelly oil	5.18	91.4 ± 1.31 ^{ab}	94.1 ± 0.69 ^b	0.38
	Castor oil	4.89	90.3 ± 0.59 ^{ab}	73.9 ± 1.52 ^c	-1.46
	unformulated	6.98	83.3 ± 0.32 ^a	90.4 ± 0.43 ^a	-1.62

(¹)Means followed by the same letter on column are not significantly different by Tukey test ($p \leq 0.05$). * Relative Virulence Index.

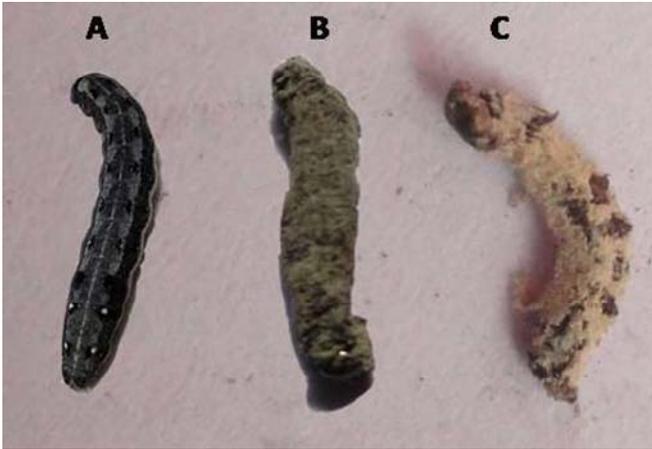


Figure 2. 3rd instar larvae of *S. litura*: A. Control larvae; B. *Metarhizium anisopliae* treated and green sporulated cadaver; C. *Beauveria bassiana* treated and white sporulated cadaver

bassiana strains. The insect cuticle contains chitin fibrils within a protein matrix together with lipids, waxes, small quantities of phenols and pigments. Oil formulations promote adherence of spores to the insect cuticle, which facilitate spore germination and subsequent infection process. Successful adhesion depends on the characteristics of mucilage, enzymes, lectins, hydrophobic bonding and electrostatic forces (Boucias et al., 1994). Spore germination is the second step of the infection process followed by formation of an appressorium and many factors have been found to play an important role in conidial germination (Butt, 1990). It is clear from the results that the oil formulations enhance the adhesion of spores to the cuticle and that the low concentration of 2% oil formulations demonstrated mortality rates up to 97% against *S. litura*.

From the present study it is evident that use of formulations at as low as 2% oil generated mortality rates of up to 93% against *S. litura* larvae. Formulations with low concentration of oil might prove to be desirable for use at field level.

Conflict of interests

The author(s) have not declared any conflict of interests.

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