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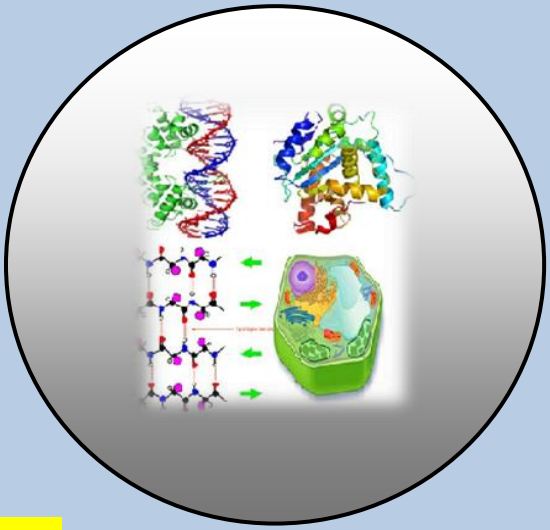

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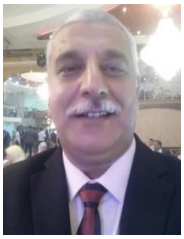

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RESEARCH PAPER

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Evaluation of Pathogenicity and Growth Regulating Potential of Certain Entomopathogenic Fungi to the Desert Locust, *Schistocerca gregaria* (Orthoptera: Acrididae)

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ABSTRACT

The efficiency of entomopathogenic fungi, Metarhizium anisopliae, Beauveria bassiana and Paecilomyces farinosus, isolated from the agricultural soil were assessed in the laboratory against the 3rd, 4th and 5th instar nymphs of the desert locust, Schistocerca gregaria (Forskal). Fungi at the concentrations 1×10^7, 2×10^7 and 4×10^7 spores/ml were applied on the desert locust by topical application. The obtained results revealed that the B. bassiana was more pathogenicity effect against 3rd, 4th and 5th nymphal instars with Lc_{50} 1.165 x 10^7, 2.665 x 10^7 and 1.998 x 10^7 spore/ml followed by M. anisopliae with Lc_{50} 3.330 x 10^7, 3.331 x 10^7 and 5.833 x 10^7spore/ml and P. farinosus with Lc_{50} 5.00 x 10^7, 8.340 x 10^7 and 13.90 x 10^7spore/ml, respectively. Of the three test entomopathogenic fungi, only B. bassiana and M. anisopliae have shown significant growth regulating effect. Conclusion from results, all tested fungi had high potentials for biocontrol agents against S. gregaria and it could be an alternative for chemical pesticides.

Keywords: Schistocerca gregaria, entomopathogenic fungi, Metarhizium anisopliae, Beauveria bassiana ,Paecilomyces farinosus, pathogenicity, mortality, development.

INTRODUCTION

The desert locust, *Schistocerca gregaria* Forskal (Orthoptera: Acrididae) is among the major insect pests due to its catastrophic damage to crops in large parts of Africa and Asia. The major control strategy adopted against the desert locust is based on the use of insecticides. The continuous use of chemical pesticides against pests has led dramatically to a resistance

to the pesticides action and causes rapid increase of insect tolerance against any type of neurotoxic insecticide Elbanna *et al*. (2012). In addition, the intensive use of these chemicals gave rise to problems such as residual toxicity (pollution) and harmful effects on beneficial insects, human beings and their domestic animals. Such problems have become a reason for searching for safe pesticides, Gabarty *et al*. (2013). Recently, the biological control, specifically, use of entomopathogenic microorganisms through their various species, easy dissemination, specificity of action and persistence in the environment is a very promising alternative to ensure effective pest control. The exploitation of entomopathogenic fungi for biological control of locust and grasshoppers has received much attention, especially in recent years (Clarkson and Charnley, 1996).

The microorganisms used in microbial control belong to several taxa namely; bacteria, viruses, fungi, nematodes and protozoa (Halouane *et al*., 2013) .Among the microorganisms used, more than 700 species of fungi are entomopathogenic agents against arthropods insects (Khan *et al*., 2012). The largest number of pathogens is in the class Zygomycetes, but most employees come from Deuteromycetes, such as *Beauveria*, *Metarhizium, Verticillium, Entomophthora and Entomophaga* (Halouane *et al.,* 2013). Like other entomopathogenic fungi, *B. bassiana* possess the potential to produce infections conidia, which penetrate the insect's cuticle, indicating that, *B. bassiana* induces on appropriate mechanism to overcome the insect's cellular defense system (Abood *et al*., 2010).

Entomopathogenic fungi are excellent candidates for use in biological control because they do not need to be ingested to cause infection; they infect their hosts by contact and then direct penetration of the cuticle (Wraight *et al*., 2000). Entomopathogenic fungi are important key factors in the regulation of insect populations in nature (Charnley, 1997). Numerous isolates of entomopathogenic fungi have been tested for their potential to control insect pests. In addition to different species and strains of entomopathogenic fungi, different formulations and application methods have also been evaluated (Langewald *et al*., 1997; Wraight and Romas,2002; Feng *et al*., 2004a, 2004b; Pu *et al*., 2005).

The entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin display a broad host range and are able to target a number of diverse arthropod groups. About 700 species of insects are known to be in the host range of *B. bassiana* (Li, 1988; Inglis *et al*., 2001) and over 200 insect species and other arthropods have also been reported that are infected by *M. anisopliae* (Cloyd, 1999).

Metarhizium anisopliae and *B. bassiana* are the most widely used entomopathogenic fungi and have been used for biological control of agricultural pests especially acridid grasshoppers and locusts in different parts of the world. Different isolates of *M. anisopliae* have been obtained from locusts and grasshoppers in Africa (Shah *et al*., 1997), Australia (Prior, 1997) and Madagascar (Delgado *et al*., 1997). Isolates of *M. anisopliae* var. acridum have been evaluated and applied to control locusts (Acrididae) under the name Green Muscle® in Africa (Douthwaite *et al*., 2000) and Green Guard® in Australia (Milner and Hunter, 2001). Among the entomopathogenic fungal products listed by Faria and Wraight (2007), *B. bassiana* constitutes (33.9%) and *M. anisopliae* also constitutes (33.9%) of total production.

The objective of this study was to evaluate the efficacy of the entomopathogenic fungi *B. bassiana*, *M. anisopliae* and *P. farinosus* on different stages of the desert locust *S. gregaria* under laboratory conditions by topical treatments.

MATERIALS AND METHODS

Insect culture

Second and third-instar nymphs of *S. gregaria* (Orthoptera: Acrididae) were collected from Baish Governorate at a distance 70km in the north of Jazan in Kingdom Saudi Arabia. The immature and mature insects of the locust were reared under the laboratory conditions of 27 ±2 ºC, 70 ± 5% RH and 12:12 daily photoperiod, according to the method described by Hunter-Jones (1966). The locust food is maize leaves and stems.

Media used for isolation of entomopathogenic fungi

For experimental studies, isolation and maintenance of stock culture, the following range of media were used.

Czapek-Dox Agar (CzDA)

Ingredients: Sucrose 30.00 g; Sodium nitrate 2.00g; Potassium dihydrogen orthophosphate 1.00g; Potassium chloride 0.50g; Magnesium sulphate 0.50 g; Ferrous sulphate 0.002g; Agar 20.00g; Distilled water 1.00 L.

Malt Extract Agar (MEA)

Ingredients: Malt extracts 20g; Peptone 1g ; Glucose 20g; Agar 20g; Distilled water 1 L.

Potato Dextrose Agar (PDA)

Ingredients: Potatoes 200 g (Blend or boil in 1L distilled Water to give slurry) ; Dextrose 15 g ; Agar 20 g; Distilled water 1 L.

Each media was prepared by dissolving the solid ingredients in one liter of cold distilled water and then heating to 60-70 ºC with stirring. Media were sterilized by autoclaving at 121 ºC for 15 minutes and then when cool to 45 ºC, it was poured into sterilized 9 mm diameter Petri-dishes for maintenance of stock cultures. Ten molten agar were poured into slants autoclaved and then tilted to provide slopes for stock cultures. The liquid medium was prepared by using the same ingredients without agar.

Collection of soil samples for fungal isolation

Surface soil samples (0-15 cm depth) were collected from underneath different field plants at Baish Governorate in the north of Jazan city. The soil samples were preserved in sterile plastic bags. Two methods used for isolation of entomopathogenic fungi , including soil (plate) method and insects bait technique.

Soil (plate) method

The samples were processed using the soil plate method (Warcup, 1950). Soil plate method: About 1g of soil was scattered on the bottom of a sterile Petri dish and molten cooled (40-45 ºC) agar medium Potato dextrose agar medium (PDA), Malt extract agar (MEA) and Czapek-Dox agar medium (CzDA) was added, which was then rotated gently to disperse the soil particles in the medium. Duplicate plates were prepared from each dilution. The plates were then incubated for 3-7 days, at 28˚C.

Insect bait method (Insect-associated fungi)

Insect-associated fungi were isolated from soil samples by using the 'Galleria bait method' (Zimmermann, 1986). The soil samples was passed through 2-mm pore sieve to remove plant tissues and molding gravels or blocks, and then mixed thoroughly and placed in glass cube (6 x 9 cm^3). The soil samples were moistened with sterile distilled water. Newely moulted 3^{rd} nymphal instar of *S. gregaria* were immersed in 56 ˚C water for 15 Second (to minimize their ability to produce skil webbing in soil (Woodring and Kaya, 1988 and Meyling *et al*., 2006) were used.

Five nymph were placed on the soil surface in glasses cube and covered with lid, and then the samples were kept at the temperature of 20–25 °C for two weeks. During the first 4 days the cubs were upended twice a day to keep the nymph moving in the soil. The cubs were examined on 7th and 14th days after inoculation, respectively. Dead nymphs were removed from the soil and surface sterilized with 3% sodium hypochlorite for 3 min and then rinsed twice with sterile distilled water. After removing free water of the nymphs surface, they were placed onto potato dextrose agar plates containing 0.1 g/L streptomycin and 0.05 g/L tetracycline. Same fungal species was only recorded once for each glasses cube, regardless isolate numbers from different nymphs.

Identification of fungal isolates

Light microscopy was used for examination and identification of the fungal isolate according to Raper and Fennel (1965) Samson *et al.,* (1995). Ellis and Ellis, 1985).

Macroscopical Investigations

The morphological features including the colour and diameter of the colony, reverse colour and sporulation were observed and recorded daily during the incubation period. For microscopical investigation, slides of fungal specimens were prepared using Cover slip technique of Kawato and Shinobu (1959) was used, the medium was poured in sterilized plate (Petri dish) and allowed to solidify, sterilized cover slips were dipped obliquely in the agar layer under a septic condition the fungal cultures were inoculated on the agar layer along the line, where the medium meets the upper surface of the Cover slip and then the cover slips were removed and fixed on slides using a tiny drop of Canada balsam, and then examined by the Image analysis system at the Regional center for Mycology and Biotechnology. Direct observations of culture, with particular emphasis on diagnostic features, were made from material mounted in distilled water. Examination was conducted with light microscope using bright field optics.

Nymphal treatment

The spores of entomopathogenic fungi were routinely grown on potato dextrose agar (PDA) media and incubated at 30±2°C till the fungi growth developed dense sporulations up to 10 days of incubation. The spores of each fungus were scraped with a spatula and kept in sterile saline water containing 0.05% of Tween 80. The desired fungal conidiospore concentration was estimated with using a haemocytometer at different concentraton ($1x10^7$, $2x10^7$ and $4x10^7$ spore/ml) and were assessed as a topical application against the 3^{rd}, 4^{th} and 5^{th} nymphal instar. The application of the fungal solution (fungal spores contained in sterilized distilled water is performed by topical application using a micropipette. A quantity of 15µl is deposited above the pronotum of all individuals experienced locusts. Control individuals were treated with sterilized distilled water. Ten locusts were used for each dose tested and the experiment was replicated three times. After 24 hours, dead locusts counted till adult emergence, also nymphal duration was recorded. Developmental duration was calculated by the Dempster's equation (1957).

Statistical analysis

LC_{50} values were calculated for mortality of each treated instar by Microsoft office Excel, 2007 according to Finny (1971). Data obtained were analyzed by the Student's *t*-distribution, and refined by Bessel correction (Moroney, 1956) for the test significance of difference between means.

RESULTS

This study was conducted to investigate the effect of entomopathogenic fungi (*B. bassiana*, *M. anisopliae* and *P. farinosus*) on the desert locust *S. gregaria*. The obtained data revealed that the entomopathogenic fungi had a lethal effect on treated nymphs irrespective of the instar and this observed by progressive mortality. Highest mortalities were recorded by *B. bassiana* followed by *M. anisopliae* and *P. farinosus*. The highest concentration (conc.) ($4x10^7$ Spore/ml) of *B. bassiana* caused 90.0, 80.0 and 73.3 % mortalities after treatment of 3^{rd}, 4^{th} and 5^{th} instar nymphs, respectively. In all treatment the lethal effect was extended to next instars and to adults. Complete nymphal mortality was recorded by the highest conc. of *B. bassiana*. However, complete mortality in all stages (nymph and adult) was recorded by *B.bassiana* only after treatment of both 3^{rd} and 4^{th} instar nymphs. No lethal effect on the emerged adults by *P. farinosus* and *M. anisopliae* after treatment of 3^{rd} and 5^{th} instar nymphs, respectively (for more details see Table 1). All entomopathogenic fungi exhibit its significant effect on the development elongation of treated nymphs except the *P. farinosus* (Table 2). After treatment of 3^{rd} instar nymphs, all conc. of *M. anisopliae* and *B. bassiana* were significantly increased the duration. The highest effect was recorded at $4x10^7$ of *B. bassiana* by 16.6±1.15 days compared to 10.7±0.82 days of control. The effect of development elongation was extended to the newly moulted 4^{th} and 5^{th} instar nymphs at conc. $4x10^7$ of *M. anisopliae* and $2x\ 10^7$ of *B. bassiana*. *P. farinosus* had no effect on the treated 3^{rd} instar nymphs but its effect was appeared only in the resulted 4^{th} instar nymphs by 15.3±1.09 and 15.7 ±1.01 days at conc. $4x10^7$ and $2x\ 10^7$, respectively compared to 12.3±1.5 days of control. After treatment of 4^{th} instar nymphs, the two highest conc. of *M. anisopliae* were significantly increased the duration and the effect was no extended to the newly moulted 5^{th} instar nymphs. However, *B. bassiana* significantly increased the duration of both treated 4^{th} instar nymphs and the resulted 5^{th} instar nymphs from the treatment. On the other hand, *P. farinosus* had no a significant effect on the duration. After treatment of 5^{th} instar nymphs, the only observed effect on the development elongation was recorded by the highest conc. of *B. bassiana* (15.6±2.00 days at conc. $4x10^7$ compared to 11.1±1.85 days of control). The results indicated that the *B. bassiana* was more effective against all treated instars nymph of *S. gregaria* as shown by the lower LC_{50} value of 1.17×10^7, 2.67×10^7 and 1.67×10^7 after treatment of 3rd, 4th and 5th instar nymphs respectively, followed by *M. anisopliae* and *P. farinosus* (for more details see Table 3 and Fig.1).

DISCUSSION

Entomopathogenic fungi are attracting attention as potential biological control agents (Clarkson and Charnley, 1996), for acridid and coleopteran pests which have no known viral or bacterial diseases. In this study, laboratory investigation was carried out to study the susceptibility of nymphal and adult stages of *S. gregaria* to the entomopathogenic fungus (*B. bassiana*, *M. anisopliae* and *P. farinosus*). Obtained results indicate that the entomopathogenic fungi had a lethal effect on treated nymphs. However, All entomopathogenic fungi exhibit its significant effect on the development elongation of treated nymphs except the *P. farinosus*.

These results agree with several studies of entomopathogenic fungi on different insect orders, against *Spodoptera littoralis,* Lepidoptera (El-Hawary and Abd El-salam, 2009), *Prostephanus truncates*, Cleoptera, by *B. bassiana conidia* (Smith *et al*., 2006).

With respect to, Orthoptera, *Schistocerca gregaria*, by *B. bassiana* and *Entomophthora sp.* (Youssef, 2014), *Tettigoniid locusts*, by *B. bassiana* and *M. anisopliae* (Mohammad Beigi and Port, 2013) and *locusta migratoria*, by Strains of *B. bassiana* isolated from locust or from the soil varied considerably in their virulence and their ability to produce in vitro toxic metabolites against *Locusta migratoria*. Among the pathogenic isolates, only culture filtrates of 90/2-Dm, 92/11-Dm and 0023-Su were toxic by injection (Quesada-Moraga and Vey, 2003).

Table 1. Mortality percentage in the 3rd, 4th and 5th nymphal instar of the desert locust *S. gregaria* treated with different concentrations of entomopathogenic fungal spores.

Entomopathogenic fungi	Conc. Spore/ml	After treatment of 3rd instar nymphs					After treatment of 4th instar nymphs				After treatment of 5th instar nymphs	
		3rd instar nymph Mort. %	4th instar nymph Mort. %	5th instar nymph Mort. %	Total Nymph Mort. %	Adult Mort. %	4th instar nymph Mort. %	5th instar nymph Mort. %	Total Nymph Mort. %	Adult Mort. %	5th instar nymph Mort. %	Adult Mort. %
Metarhizium anisopliae	$4x10^7$	70	20	0	90	33.3	53.3	20	73.3	25	40	0
	$2x10^7$	50	23.3	0	73.3	0	40	16.7	56.7	0	30	0
	$1x10^7$	26.7	13.3	0	40	0	30	16.7	46.7	0	20	0
Beauveria bassiana	$4x10^7$	90	10	-	100	-	80	16.7	96.7	100	73.3	75
	$2x10^7$	66.7	16.7	6.6	90	100	53.3	20	73.3	62.5	40	41.7
	$1x10^7$	50	13.3	10	73.3	100	36.7	16.6	53.3	42.8	26.7	25.0
Paecilomyces farinosus	$4x10^7$	43.3	20	10	73.3	0	30	13.3	43.3	11.7	20	0
	$2x10^7$	33.3	16.7	3.3	53.3	0	20	16.7	36.7	0	10	0
	$1x10^7$	20	10	6.6	36.6	0	13.3	10	23.3	0	13.3	3.7
control		0	5	0	5	0	0.0	3.3	3.3	0	0.0	0

No. of tested nymph=30; Conc.=Concentration; Mort.= Mortality

Also, our results in consistent with Lednev *et al*. (2008), when they tested *B. bassiana* and *M. anisopliae* against migratory locust *Locusta migratoria* and they found four or five day after the *L. migratoria* had been infected with *B. bassiana* and *M. anisopliae* Sorokin a sharp increase in nymphas mortality was observed reaching 95-100% on the 13th to 17th days after inoculation. However, for grasshoppers and locusts, Bateman (1997) reported that insect field mortality caused by pathogen rarely occurs earlier than 6 days after application.

In a field trial the fungal product *M. anisopliae* var. acridum strain IMI 330189 developed by the LUBILOSA project, showed the first observable mortality of the acridid species at7-10 days after application and the full effects were observed 14-18 days after application (Lomer *et al*., 1997).

Table 2. Effect of various concentrations of entomopathogenic fungal spores on the developmental duration of the 3rd, 4th and 5th nymphal instar of the desert locust *S. gregaria*.

Entomopathogenic fungi	Conc. Spore/ml	After treatment of 3rd instar nymphs			After treatment of 4th instar nymphs		After treatment of 5th instar nymphs
		Duration of 3rd (days)± SD	Duration of 4th (days)± SD	Duration of 5th (days)± SD	Duration of 4th (days)± SD	Duration of 5th (days)± SD	Duration of 5th (days)± SD
Metarhizium anisopliae	$4x10^7$	14.6±1.06 c	15.3±0.58 b	16.0±1.00 b	13.1±1.09 b	14.7±1.30 a	14.2±1.72 a
	$2x10^7$	14.2± 0.84 c	14.4± 0.74 a	14.6±1.2 a	13.4±1.52 b	14.9±1.41 a	14.3±1.98 a
	$1x10^7$	13.4±1.40 b	13.0±1.30 a	13.8±1.4 a	10.9±1.74 a	13.5±1.57 a	13.6±2.00 a
Beauveria bassiana	$4x10^7$	16.6±1.15 c	-	-	15.6±0.89 b	17*	15.6±2.00 b
	$2x10^7$	15.0±0.82 c	15.2±0.84 b	15.7±0.58 b	16.3±1.53 c	17.8±1.72 b	14.5±2.10 a
	$1x10^7$	15.7±1.03 c	14.8±0.94 a	15.1±1.06 a	13.5±1.72 b	16.5±1.72 b	14.6±1.95 a
Paecilomyces farinosus	$4x10^7$	12.5±1.11 a	15.3±1.09 b	13.2±1.51 a	10.3±1.57 a	12.8±1.41 a	12.5±2.10 a
	$2x10^7$	12.9±1.34 a	15.7 ±1.01 b	12.7±1.43 a	9.7±1.89 a	12.4±1.76 a	11.6±2.00 a
	$1x10^7$	11.1±1.56 a	14.8±1.30 a	12.9±2.1 a	9.4±1.61 a	11.1±1.83 a	11.7±1.90 a
Control		10.7±0.82	12.3±1.5	11.6±2.0	8.9±1.69	11.6±1.77	11.1±1.85

Conc.: concentration, mean ± SD followed with the letter (a): is not significantly different (P>0.05), (b): significantly different (P<0.05), (c): highly significantly different (P<0.01), *: one insect.

Table 3. LC_{50} values (spore/ml) of entomopathogenic fungal spores against 3rd , 4th and 5th nymphal instar of the desert locust *S. gregaria*.

Entomopathogenic fungi	LC_{50} values (spore/ml)		
	After treatment of 3rd instar nymphs	After treatment of 4th instar nymphs	After treatment of 5th instar nymphs
Metarhizium anisopliae	3.330×10^7	3.331×10^7	5.833×10^7
Beauveria bassiana	1.165×10^7	2.665×10^7	1.998×10^7
Paecilomyces farinosus	5.000×10^7	8.340×10^7	13.90×10^7

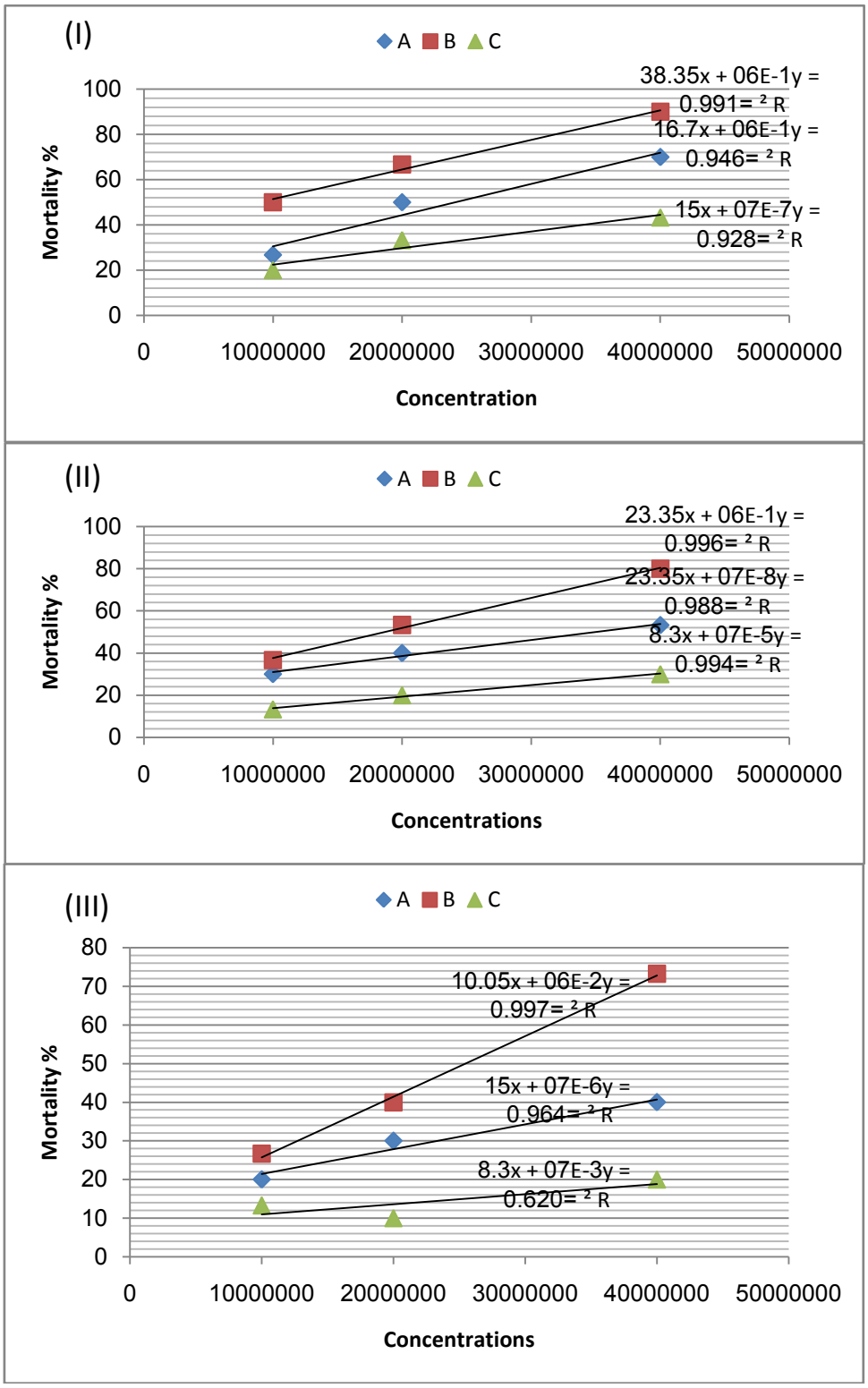

Fig. 1. Regression Line of desert locust mortality after treatment *by Metarhizium anisopliae* (A), *Beauveria bassiana* (B) and *Paecilomyces farinosus* (C). Where: (I), after treatment of 3rd instar nymphs, (II), after treatment of 4th instar nymphs, (III), after treatment of 5th instar nymphs.

From the above results, the percentages could be due to impact strength and inactivation of the immune system or by the production of insecticidal cyclodepsipeptides of the group of destruxins as in the strains of *M. anisopliae* (Kershaw *et al*., 1999; Amiri-Besheli *et al*., 2000). Milat-Bissaad *et al*. (2011) noted that the decrease in haemocytes of *S. gregaria* resulted in depletion of reserves accumulated in the fat body. The decrease in haemocyte numbers in response to mycosis of *S. gregaria* caused by *B. bassiana* and *M. anisopliae* var. *acridum* may be due to the intervention of these haemocytes in autophagy and humoral defense reactions and these lead to the nymphs mortality of *S. gregaria*. Other studies have shown the increasing of the hemocytopoitic activity as long as conidia dosage. However, the fungus circumvents the host's immune defenses by preferentially destroying prohaemocyte and plasmatocytes, the most common haemocytes types (*Zootermopsis angusticollis*) Avulova and Rosengaus (2011).

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