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Lab-Field Evaluation of Some Egyptian Isolates of Entomopathogenic Fungi *Metarhizium Anisopliae* and *Beauveria Bassiana* Against Sugar Beet Beetle *Cassida Vittata* Vill.

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Soil samples from different governorates i.e., Elbehaira (119 samples), Kafr El-Shaeikh (103 samples) and Aswan (97 samples) were investigated. Only 10 samples (3.134 %) contained entomopathogenic fungi, i.e., 7 isolates contain *Beauveria Bassiana* and 3 isolates contain *Metarhizium Anisopliae*. The fungi were propagated on PDA media and rice grains. PDA media gave the highest Conidiophores production by *B. Bassiana* and the rice media gave the highest Conidiophores by *M. Anisopliae*. Bioassay for the determination of LC_{50} values took place versus larvae of the greater wax moth and *Galleria mellonella*. Conidia were prepared in the lab. for bioassay and filed application at the concentrations of 1.3×10^3 ,1.3×10^4 and 1.3×10^5 spores/ ml. Results were obtained to confirm successful control against larvae and adult populations of the sugar-beet beetle, *Cassida vittata* Vill. Results indicated that the fungi, *M.anisoplia*, gave a good effect on *Cassida vittata* larvae than *Beauveria bassiana* in the two experimental seasons. In lab-experiments, the same trend obtained by LC50 and LT50 recorded. The fungi, *B.bassiana* spores tolerate the ambient atmosphere (temperature) than the *M.anisoplia*.

Keywords: Sugar-beet, Beauveria bassiana, Metarhizium anisopliae, Cassida vittata, biological control

INTRODUCTION

Sugar beet, *Beta vulgaris* L. Is the second strategic sugar crop after the sugar cane in Egypt. Because of its lower consumption of irrigation water and its shorter growing season, sugar beet is planted on extending area, especially, with a decreasing area of the sugar cane crop which needs water and long season (nine months) in Egypt. Sugar beet plants attract a considerable number of insect pests, among them and most importantly is the tortoise beetle, *Cassida vittata* (Coleoptera: Chrysomelidae) (Mahmoud et al. 1973; Youssef 1994;Saleh et al., 2009). Larvae and adults of *C. vittata* are leaf feeders. Crop loss occurs due to leaf feeding and a reduction in sugar content of infested plants (Aly et al. 1993; Al-Habshy, 2013).

This pest is controlled by conventional chemical insecticides in Egypt. Interest in the use of foliar fungicides for sugar beet and other crops has expanded dramatically in the world over the past few years. Applications for the purpose of protecting crop yield were rarely economical. Several factors have played a role in the escalation of fungicide use on the different crops. The best chance that a fungicide treatment will result in a net economic gain for sugar beet occurs when disease conditions exist which justify making an application. Be cautious, fungicides provide protection for a limited time and if applied prematurely, they will lose their effectiveness by the time the disease actually makes its appearance. Some producers ask if an insecticide should be included with the fungicide application in place of an adjuvant to provide better distribution of the fungicide on plants, as well as to protect against possible insect damage.

At the present time, the environmental contamination reached high rates due to excess use of chemical pesticides in controlling pests. Recently, researchers tend to use botanical pesticides and microbial entomopathogenic fungi in controlling pests and these are mostly safe for humans and the environment (Migiro et al. 2011; Abd El-Salam et al.2012&2013). The aim of the present work is to investigate and evaluate the efficacy of some Egyptian entomopathogenic fungi isolations against the tortoise beetle, *Cassida vittata*.

MATERIALS AND METHODS

Collection of soil samples

The study material consisted of soil samples taken at the beginning of December 2011 in three localities, Elbehaira, Kafr Elshaeikh, and Aswan Governorates. Samples were taken at random, separately for each Governorate from 10-15 sites in the test areas. The material was collected using Egner's stick

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to a depth of 15 cm. A mixed sample was prepared from the collected material and stored in plastic bags (ice box) at 0-4°C. Immediately before starting the isolation processes in the laboratory, the soil was sieved and dried up to a suitable moisture content, optimal for fungal growth and limiting the development of nematodes. Soil samples from all localities were analyzed to assess their granulometric composition (soil type), pH and organic carbon content.

Isolation and Identification of Fungi

Entomopathogenic fungi were isolated from soil using two methods: the insect bait method proposed by Zimmermann (1986) and the selective medium method developed by Strasser et al. (1996) and commonly used for the isolation of entomopathogenic fungi from soil (Keller et al. 2003). The Greater Wax Moth (*Galleria mellonella* L.) was used as a bait insect. Ten plastic boxes with a capacity of about 200 ml were filled with soil from each governorate. Ten *Galleria mellonella* larvae were put in each box, a total of 100 larvae. The boxes of soil were placed in an incubator at 20-22°C.

The first mortality recorded was conducted 7 days after the start of the experiment and then at 3-day intervals until the death of all larvae. Dead larvae were washed in distilled water and then surface-sterilized for 30 seconds in 1% sodium hypochlorite solution. After rinsing twice in distilled water, the larvae were put on Petri dishes with moistened filter paper. The dishes with the larvae were kept at 20-22°C in the dark conditions. Fungi growing on insects were transferred on standard media and then examined microscopically. Identification of isolated fungal species was done by using standard methods described by Goettel and Inglis (1997). Fungal nomenclature follows Sung et al. (2007) and Index Fungorum (www.indexfungorum.org).

Additionally, the concentration of colony-forming units (CFU) of entomopathogenic fungi in soil environments tested was determined applying a selective medium developed by Strasser et al. (1996). For this purpose, 2.0 gm of soil was weighed out from each mixed sample originating from a given locality, and then 18 ml of distilled water was added with the addition of 0.05 Triton X-100, and the components were vigorously shaken for about 35seconds. Then 0.1 ml of the soil solution was poured out and spread using a glass spatula on a selective medium in four Petri dishes which were the replicates.

The dishes were placed in incubators at 22° C and after 8-10 days colonies of individual fungal species were counted. The results were expressed as the number of colony-forming units (CFU) of entomopathogenic fungi in 1.0 g of the soil. The selective medium used, had the following composition: 10 g of peptone, 20 g of glucose and 18 g of agar were added to 1.0 liter of water. After sterilization and cooling, the following components were added to the medium: 0.6 g of streptomycin sulfate, 0.05 g of chlortetracycline, 0.05 gm of cycloheximide and 0.1 gm of dodine.

Insect Rearing

Larvae of *C. vittata* were collected from sugar beet fields (El-Behera Governorate, North-west Egypt) and transferred to the National Research Center laboratory. Larvae were fed on fresh leaves of sugar-beet plants inside glass jars and left to reach the pupal stage and then the adult stage. Newly fifty pairs adults (5 old days) were left to mate, then the eggs were collected and left to hatch. The 1st larvae resulted were reared on fresh leaves of sugar-beet plants till the fourth instar and adult stage. The temperature and the relative humidity during the rearing experiment were 25.0 ± 1.0˚C and 65.0± 5.0 % RH. (Abd El-Raheem, 2000).

Preparation of the Concentrations

Spores of fungal isolates harvested by rinsing with sterilized water, 0.5 % Tween 80 from 14 day old culture (PDA) media. The suspensions were filtered through cheese cloth to reduce mycelium clumping. The spores were counted in the suspension using a haemocytometer (0.1 mm x 0.0025 mm^2). One ml from the suspension contained 1.3x 10^3 Spores.

Bioassay Procedures

Fourth instars larvae of *Cassida vittata (5 old days)* were treated with conidiospores of *B. bassiana* and *M. anisopliae* with the following concentrations 0.5, 1.0 and 2.0 ml. Fifty larvae transferred in groups. Five individuals placed in Petri-dish on a wetted filter paper. After dipping the leaves of sugar-beet (2.5 cm diameter) for five seconds in the suspension, the larvae and the leaves were transmitted to ~~the~~ another dry Petri-dish. Each treatment was incubated at 25.0 ±2.0˚C and 85.0± 5.0 % RH. Treatment was observed and mortality counted daily. Control leaves were treated with distilled water. Each treatment contained five replicates. Lethal concentrations ($LC_{50,90}$) and lethal time ($LT_{50,90}$) were estimated by Probit analysis (Finney, 1971)

Field Applications

Achieving good results with fungicides requires excellent spray coverage, regardless of the crop. Timing is also critical. Fungi have certain life stages that are vulnerable to fungicides.
Beauveria bassiana and *Metarhizium anisopliae* application were carried out in Elbehaira. Identical trials were conducted at the NRC of nubaria experimental station, during mid February to mid March 2012 and the third week from February to the third week of March 2013. Sugar-beet plants were sprayed with the fungal suspensions to evaluate its efficacy against the larvae and the adults of *C. vittata.* Conidia spores of *Beauveria bassiana* and *Metarhizium anisopliae* were applied to sugar-beet plants by using the concentrations of 0.5, 1.0 and 2.0 ml/liter water (1.0 ml from the suspension contained 1.3x 10^3 Spores).

An area of about one feddan (4200m^2) was divided into thirty five plots (five plots /treatment/concentration were treated with *B. bassiana* and *M. anisopliae*) and the other five plots sprayed with water (control). Agriculture practices were performed, without any pesticide treatments. The suspension was sprayed four times a month. Twenty plants from each replicate were collected and inspected to count the number of alive larvae, before and after each spray. The suspension sprayed early in the morning, and five replicates were determined. Mortality data were analyzed with one-way analysis of variance (ANOVA) and the least significant difference (LSD) test was used to compare means. Statistical analyses were performed using SPSS 8.0 for Windows (SPSS, 1997).

RESULTS AND DISCUSSION

Isolation Processes

Isolates of B. bassiana and Metarhizium anisopliae selected for screening originated from diverse sites in Egypt (Table 1); there were six isolates from different soil samples. Three isolates of B. bassiana and other for Metarhizium anisopliae. Isolates of Beauveria bassiana were coded as B1E, B2E, and B3E from Elbehaira, B4K and B5K from Kafr El-Shaeikh and B6As from Aswan. Similarly, the new isolates of Metarhizium anisopliae were coded as M1E, from Elbehaira, M2K, and M3K from Kafr El-Sheikh and M4As from Aswan.

Laboratory Studies

The number of samples collected from different Governorates, in which represent moderate temperature (Behera & Kafr El-Shaeike), and the third Governorate represent the hot environment (Aswan). The samples in which investigated and evaluated, clearly indicated that the atmospheric temperature has direct effect directly on soil temperature, in which affects on the viability of fungi spores. Table (1) shows that from the total samples inspected (total samples 319), only six samples (El-behera 3 samples; Kafr-El-shaeike, 2 samples and one sample in Aswan governorates, these samples represent 1.88% from the total samples) contain B.bassiana. In another side, the samples only 4 samples contain M.anisopliae only 4 samples (El-behera 1; Kafr-elshaeike, 2 and Aswan, 1).

From the obvious counts, it can be revealed that the fungi B.bassiana spores tolerate the ambient atmosphere (mainly temperature) than the M.anisopliae spores. Table (2) shows some interesting investigations: the first one, is that the increase of concentrations, decreases the LT50 and LT90 for the two isolate fungi. Statistical analysis indicates that there is no significant differentiation recorded between the three governorates under our studies for the fungi, M.anisopliae . For the fungi, B.bassiana, to receive good results, 2.0 ml should be used for the optimal condition can be used 2.0 ml. (LT50 5.6193 and 6.8437).

While in case of M.anisopliae , the LT50 ranged between 4.8232 to 5.1386. Concerning For obtaining high effect (LT90), the suitable concentration is 2.0 ml. suspension. The results recorded indicate that, there is no differentiation between the three Governorates for M.anisoplia, while for B.bassiana , the best isolate is B4As (Table2). Data illustrated from Table(3) shows that the LC50 and LC90 for the two fungi isolated, indicate that the LC50 for (M.anisoplia) were 0.2432 (M1 E); 0.3064 (m2 k) and 0.3411 (M4 As), while LC50 for B.bassiana were 0.0993 (B1 E); 0.232 (B2 K) and 0.4415 (B4As). For LC90 the data indicated that the best isolate for M.anisoplia is M1 E and B2 K for B.bassiana .

Field Studies

Season 2012

From The data recorded in Table(4) indicated that the population of target pest larvae in all plots ranged between 4.0 and 3.6 larvae/plant just before spray. After one week from the spraying with the, M. anisopliae suspension, the results recorded a high level of larval reduction (48.6 to 82.0%), while B.bassiana had a less percentage (36.9 to 68.5%). After two weeks from the beginning of experiments, the results recorded, indicated that the percentage of alive larvae reduction increased gradually and correlated with the concentration rates. Table (4) indicates that the percentage of larval reduction increased gradually till the 4th spray, which reached 97.3% (M.anisoplia, M4 As) while the percentage of larval population reduction was 92.9 (B.bassiana, B6As). From the same Table (4), the average percentage of reduction reached 90.1 % (M.anisoplia, M4As (2012) and In 2013 season (Table5) F. test indicates that there is significant difference between the two pathogens and its concentrations. LSD50 explains that the time is a very important factor to observe the effect of any pathogen. After 4th spray, from the beginning of application, the percentage of alive larvae reduction ranged between 84.0 to 92.9 % and 86.5 to 97.3% for B.bassiana and M.anisopliae respectively.

Season 2013

Table (5) revealed that there is no significant difference between the efficacy of the two pathogens isolated at the beginning of application to the end of experiments after 4th spray, except some concentrations, but in all the data recorded indicated that generally, the environmental conditions observed in the two seasons, play a role for in the differentiation of data recorded. Comparing the main average of the percentage for larval reduction, the range was between 59.5 to 83.6 % and 63.2 to 82.25 % at 2012 and 2013 respectively for (B.bassiana) while for M.anisoplia , it ranged between 71.3 to 90.1 % (2012) and 77.4 to 85.6 % (2013). The difference between the two successive season's data is due to the change in the environmental conditions.

DISCUSSION

Environmentally friendly biopesticides control can play a great role in sustainable crop production by providing successful pest management. Data from these studies, indicated that the use of entomopathogenic fungi, B. bassiana; M. anisopliae reduced the number of alive larvae of the target pest. Tests with B. bassiana and M.anisopliae have given promising results for the control of C. formscarius in India (Tarafdan and Sarkar 2006; Ondiaka et al. 2008.While adults are the only noticeable stage, infected adults can transmit infestations to other individuals in the field. The field efficacy of entomopathogenic fungi, towards different pests depends on many factors, such as behavior of the pest (Gindin, et al. 2006). Although the adults feed on plant canopy they can be seen crawling on the soil where it is possible that they become contaminated with the fungal spores. This study, showed the potential of entomopathogens as an alternative to the currently employed traditional insecticides. When foliar disease pressure is severe, the blighted leaves cannot produce enough photosynthates (sugars) to adequately fill the roots.

Table (1): Numbers of positive entomopathogenic fungi recovery by trapping with larvae of *G. mellonella* from soil samples collected from three governorates and their rates of incidence (%)

Locality	Number of samples	Fungi positive samples and incidence (%)	
		B. bassiana	***M. anisopliae***
Elbehaira	**119**	**3 (2.52%)**	**1 (0.84%)**
Kafr El-Shaeikh	**103**	**2 (1.94%)**	**2 (1.94%)**
Aswan	**97**	**1 (1.03%)**	**1 (1.03%)**
Total	**319**	**6 (1.88%)**	**4 (1.25%)**

Table (2): Estimates of the $LT_{50\&90}$ (days; all replicates combined) for *C. vittata* larvae exposed to 3 different isolates of *M. anisopliae* and *B.bassiana.*

Species	Fungi isolation	Conc. (ml)	LT_{50} (days)	LT_{90} (days)	X^2
M. anisopliae	M_1E	0.5	6.7985	11.3321	0.7866
		1.0	5.925	10.4041	4.1402
		2.0	4.8919	7.4833	1.5231
	M_2K	0.5	7.1121	12.3975	5.6333
		1.0	6.3915	10.8095	4.8075
		2.0	4.8232	7.6662	2.3988
	M_4AS	0.5	6.1384	9.8402	12.5462
		1.0	5.7903	9.2065	7.9547
		2.0	5.1386	6.9377	1.3417
B.bassiana	B_1E	0.5	10.542	19.9373	1.2046
		1.0	10.6185	25.4406	0.1377
		2.0	6.8437	17.0108	0.2881
	B2K	0.5	10.6296	19.2246	0.9325
		1.0	10.1018	22.0639	3.5357
		2.0	6.4239	17.6017	0.7872
	B4AS	0.5	8.4825	15.8643	1.4382
		1.0	7.8385	15.586	1.2141
		2.0	5.6193	11.4752	1.0947

Table (3): Estimates of the $Lc_{50\&90}$ for *C. vittata* larvae exposed to 3 different isolates of *M. anisopliae* and *B.bassiana.*

Species	Fungi isolation	LC_{50}	LC_{90}	X^2
M. anisopliae	M_1E	0.2432	1.4589	0.0687
	M_2K	0.3064	2.1422	8.9321
	M_4AS	0.3411	1.1958	26.1545
B.bassiana	B_1E	0.0993	2.807	6.8265
	B_2K	0.2632	1.805	14.9941
	B_4AS	0.4415	6.4684	29.2979

Table (4): Efficacy of the Fungi isolations against *C. vittata* larvae in sugar beet field during 2012season.

Fungi isolation	Conc. (ml)	Mean No. of alive larvae / plant & % Reduction after 4th applications during four weeks									Avg. % Red.
		1st spray			2nd spray		3rd spray		4th spray		
		Population before 1st spray	Population after one week	% Red.	Population after two week From spray	% Red.	Population after three weeks	% Red.	Population after four weeks	% Red.	
B. bassiana (B_6As)	0.5	4.0a	3.0b	36.0	2.2b	64.7	2.0b	52.7	1.6b	84.0	59.35
	1.0	3.8a	2.2bcd	51.0	1.6b	73.0	1.2bc	83.8	1.0b	88.2	74.0
	2.0	3.8a	1.4cde	68.5	1.0b	83.1	0.8c	89.8	0.6b	92.9	83.6
M. anisopliae (M_4As)	0.5	4.0a	2.4bc	48.6	2.0b	67.9	1.4bc	82.0	1.2b	86.5	71.3
	1.0	4.0a	1.2de	74.3	1.2b	80.8	0.8c	89.8	0.4b	95.5	85.1
	2.0	3.8a	0.8e	82.0	0.8b	86.5	0.4c	94.6	0.2b	97.3	90.1
control	0.0	3.6a	4.2a	-----	5.6a	-----	7.0a	-----	8.0a	-----	------
F		0.02 un	8.9*		10.87*		38.9*		21.5*		------
$LSD_{0.05}$		2.65	1.13		1.44		1.65		1.7		------

Treatment means followed by the same letter are not significantly different from each other (P < 0.05).

Table (5): Efficacy of the Fungi isolations against *C. vittata* larvae in sugar beet field during 2013 season.

Fungi isolation	Conc. (ml)	Mean No. of alive larvae / plant & % Reduction after 4th applications during four weeks									Avg. % Red.
		1st spray			2nd spray		3rd spray		4th spray		
		Population before 1st spray	Population after one week	% Red.	Population after two week From spray	% Red.	Population after 3 weeks	% Red.	Population after 4weeks	% Red.	
B. bassiana (B6As)	0.5	3.0a	2.6ab	41.0	2.0b	56.7	1.6b	69.9	1.0b	85.1	63.2
	1.0	3.0a	2.0ab	55.0	1.6bc	65.4	0.6c	88.7	0.6b	91.1	75.8
	2.0	3.0a	1.6ab	64.0	1.0bc	78.4	0.4c	92.5	0.4b	94.1	82.25
M. anisopliae (M4As)	0.5	3.0a	2.0ab	54.4	1.0bc	78.4	0.6c	88.7	0.8b	88.1	77.4
	1.0	3.0a	1.6ab	63.6	0.8c	82.7	0.6c	88.7	0.4b	94.1	82.3
	2.0	2.6a	1.2ab	68.5	0.6c	85.7	0.4bc	91.4	0.2b	96.6	85.6
control	0.0	2.6a	3.8a	-----	4.0a	-----	4.6a	----	5.8a		----
F		0.08un	2.96un		10.05*		19.7*		18.42*		
$LSD_{0.05}$		1.93	1.45		1.07		0.17		1.09		

Treatment means followed by the same letter are not significantly different from each other (P < 0.05).

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