Isolation, Propagation and Application of the Entomopathogenic Fungi for Controlling Noctuid and Cassidid Pests in Sugar Beet

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ABSTRACT

Out of 1491 soil samples from different governorates, *i.e.*, Giza (418 samples), Dakahlyia (683 samples) and Kafr El-Sheikh (390 samples) only 16 samples (1.07%) contained entomopathogenic fungi, *i.e.*, 9 isolates from *Beauveria bassiana*, 10 isolates from *Metarhizium anisopliae*, and 5 sampleas from *Paecilomyces lilacinus*. The fungi were propagated on PDA medium fortified with nutritional sources of plant origin, *e.g.*, crushed grains / seeds or molasses. The addition of 100g crushed rice grains/1L of PDA gave the highest conidiospore production by *Beauveria*, crushed wheat grains for *Metarhizium* and crushed corn for *Paecilomyces*. Bioassay for determination of LC_{50} values took place versus larvae of the greater wax moth, *Galleria mellonela*. Conidia were prepared in EC formulation for field application at the concentration of $1x10^6$ spores/ml. Results showed successful control against larval population of the cotton leafworm, *Spodoptera littoralis*, the beet worm, *S. exigua*, the semi-loopers, *Autographa gamma*, *Syngraphga circumflexa*, and *Phytometra ni*. Although the laboratory treatment resulted high mortality rates, but it was not successful in the field when sprayed against immature stages and adults of the tortoise beetle, *Cassida vittata* inhabiting the lower leaf surfaces where they escaped the sprayed conidiospores.

Key Words: *Beauveria bassiana, Metarhizium anizopliae, Paecilomyces lilacinus* isolation, propagation, microbial control, semi-loopers, *Spodoptera littoralis, Cassida vittata.*

INTRODUCTION

As an alternative to chemical control or as part of IPM programs, there is a resurgence of interest in the use of microbial insecticides for biological control of insect pests (Castillo *et al.*, 2000). Particularly, the entomopathogenic fungi have long been known to cause epizootics among certain insects under both laboratory and field conditions (Barson *et al.*, 1994; Watson *et al.*, 1996; Reithinger *et al.*, 1997). Traditionally, chemical pesticides control the sugar beet insect pests in Egypt. The sugar beet planted area is expanding and new fabrics for production of the beet sugar are constructed in the new cultivated areas. A project for controlling the sugar beet insect pests with entomopathogenic fungi was funded by the Ministry of Agriculture in 2001 contributing to the policy in supporting IPM strategies in different economic crops.

The present study represents the obtained results in this project concerning isolation of entomopathogenic fungi from soil samples. Also, PDA medium was improved by adding different crushed grains and seeds to reach the highest production of conidiospores. The produced conidia of *Beauveria bassiana* (Bals.) Vuillemin were bioassayed versus larvae (L_4) of the greater wax moth, *Galleria mellonella* L. Five sprayings ($1x10^6$ conidiospores/ml) were applied in a sugar beet field in 2002 season for controlling the cotton leafworm, *Spodoptera littoralis* (Boisd.), the semi-loopers, *Autographa* gamma, *Syngrapha circumflexa*, *Phytometra ni*, and the tortoise beetle, *Cassida vittata* Vill.

MATERIALS AND METHODS

Isolation of Entomopathogenic Fungi

1491 soil samples were collected from Giza (418 samples), Kafr El-Sheikh (390 samples), and Dakahlya (683) samples for trapping the entomopathogenic fungi following the technique created by Zimmermann (1986) using larvae of the greater wax moth, *Galleria mellonella* L. as highly sensitive trapping insect for pathogenic fungi and entomogenous nematodes. Also, trapping with larvae and adults of the flour beetle, *Tribolium confusum* was followed.

Bioassay Technique

Seven concentrations of spore suspensions of 10, $1x10^2$, $1x10^3$,$1x10^4$, $1x10^5$,$1x10^6$, and $1x10^7$ spores/ml were tested versus *G. mellonella* L_4 larvae of by direct spray technique using 4 replicates each of 25 larvae, and versus immature feeding stages of the sugar beet key insect pests in 4 replicates each of 20 individuals. LC_{50} and LC_{90} values were calculated. Using small perfume atomizers, the spore suspensions were directly sprayed onto the test insects.

Production of the Entomopathogenic Fungi

The isolated fungi were primarily purified using the monospore technique. They were propagated in Petri-dishes (10 cm ø) on potato dextrose agar medium (PDA) enriched with 1% peptone, 4% glucose and 0.2% yeast and incubated at 26°C. Seven-day old cultures with well developed spores were harvested by washing with 10 ml sterilized distilled water + 0.03% Tween-80 and used as stock suspension with known spores count and kept in refrigerator at 4°C, from which the fungi were subcultured or produced for laboratory evaluation tests (infectivity and bioassay tests) or adjusted in conidiospore concentration of $1x10^6$/ml and mixed with 1% sunflower oil for field application using the spraying technique. Culturing produced large amounts of conidiospores on liquid medium (Rombach *et al.*, 1988) in conical flasks or in 1L

cell culture glass bottles or on rice grains in 0.5 kg marmalade jars.

Field Application Technique and Dates

One feddan planted with sugar beet was available in Sakha Experiment Station, Kafr El-Sheikh governorate in 2002 season, and half feddan in 2003 season beside 8 feddans in Fayoum in 2003 season to carry out the first application trials for controlling the sugar beet insect pests with the entomopathogenic fungi. Twelve visits to the experimental fields in Sakha were arranged from the beginning of 7/11/2001 to 11/5/2002 for monitoring the populations of the different insect pests on sugar beet leaves. During this period, 5 applications by different spraying techniques were carried out as follows: the *1st* (7/11/2001) and the *2nd* (25/3/2002) using a 5-L sprayer, the *3rd* (01/4/2002) by a 10-L sprayer, the *4th* (13/4/2002) by a 10-L spraying motor, and the *5th* (20/4/2002) by a 600-L spraying motor. All applications were carried out with a concentration of $1x10^6$/conidia/ml. The *M. anisopliae* isolate M7D was applied in the *4th* treatment, while the *B. bassiana* isolate B7D was used for the other applications.

Larvae of the lepidopterous pests and eggs, larvae, pupae and adults of the tortoise beetle were counted just before fungi application and one week later. Monitoring of pests in sugar beet experimental field was carried out on random samples of 20 plants from the treated plots as well as from the untreated control. The formula of Hendreson and Tilton (1955) was used to calculate the reduction rate among populations of the targeted sugar beet pests in the field after application with the entomopathogenic fungi.

RESULTS AND DISCUSSIONS

Isolation of Entomopathogenic Fungi

As seen in Table (1), recovery percentages for the fungus *B. bassiana* from soil samples were 0.47, 0.29 and 0.25%; for *M. anisopliae* 0.47, 0.29, and 0.51%; and for *P. lilacinus* 0.47, 0.29, and 0.25% among those collected from Giza, Dakahlya, and Kafr El-Sheikh governorates, respectively. The total recovery percentages for the three successive fungi were 33, 42, and 33%, representing a total recovery rate of 1.07% from all collected soil samples.

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

Locality	No.	Fungi +ve samples and incidence (%)		
		Beauveria	*Metarhizium*	*Paecilomyces*
Giza	418	2 (0.47%)	2 (0.47%)	2 (0.47%)
Dakahlya	683	2 (0.29%)	2 (0.29%)	2 (0.29%)
K. El-Sh.	390	1 (0.25%)	2 (0.51%)	1 (0.25%)
Total	1491	5 (0.33%)	6 (0.42%)	5 (33%)
			16 (1.07%)	

Bioassay of isolates

Selected isolates were bioassayed versus larvae (L_4) of the greater wax moth, where calculated LC_{50} and LC_{90} values are presented in Table (2). Bioassay studies resulted different values in the LC_{50} and LC_{90} between the three genera of the isolated entomopathogenic fungi, *i.e., Metarhizium, Beauveria,* and *Paecilomyces*. The three *Beauveria* isolates (B7D, B8D and B9K) showed the same LC_{50} ($2x10^5$ spores/ml). Meanwhile, the *Metarhizium* isolate M8D was more effective at both levels of LC_{50} ($1x\ 10^4$) and LC_{90} ($7x\ 10^7$) than the isolates M7D ($2x10^4$ and $1x10^8$) and M9K ($2x10^4$ and $2x10^8$) and selected for one application in the season. For *Paecilomyces,* the isolates P7D and P8D were similarly effective (with LC_{50} of $1x10^8$) than P9K ($2x10^8$). Hluchy (1989) reported similar results, while Draganova (1998) recorded higher efficacy for blastospores than using conidia when bioassayed versus *G. mellonella* larvae (L_3).

Table (2). Values of LC_{50} and LC_{90} for the selected entomopathogenic isolates versus larvae (L_4) of *G. mellonella.*

Fungus	Isolates	LC_{50}	LC_{90}
B. bassiana	B7D	$2x10^5$	$1x10^{10}$
	B8D	$2x10^5$	$5x10^9$
	B9K	$2x10^5$	$5x10^{10}$
M. anisopliae	M7D	$2x10^4$	$1x10^8$
	M8D	$2x10^4$	$7x10^7$
	M9K	$2x10^4$	$2x10^8$
P. lilacinus	P7D	$1x10^8$	$1x10^{19}$
	P8D	$1x10^8$	$2x10^{19}$
	P9K	$2x10^8$	$6x10^{19}$

As these results show nearly no differences between the efficacy of some isolates of the same entomopathogenic fungus species versus larvae of the wax moth, *G. mellonella.* It is needed to observe any difference between such isolates using electrophoretic techniques for protein or DNA identification.

Results of Field Application

The Cotton Leafworm, *S. littoralis:*

Data presented on Fig. (1); and Table (3) showed that the *1st* application with *B. bassiana* (isolate B7D) decreased the population of *S. littoralis* by 50% after 20 days from application among the so called autumn generation, where the plant leaves are still small in size. During the late winter and early summer generations, four successive applications at one-week intervals were sprayed. The second *B. bassiana* application (25/3/2002) caused reduction of 63.6% among larvae of *S. littoralis* one week after application, that remained stable (63%) after the third application (1/4/2002). Using *M. anisopliae* in the *4th* application (13/4/2002) decreased larval population by 62.5%; relatively the same effect of *B. bassiana.* The *5th* application caused 100% reduction one week after treatment. Many authors recorded successful laboratory control tests with the two fungi against *Spodoptera* spp. and other noctuids, *e.g.*, Fargues and Rodrigues-Rueda (1980), Maniana and Fargues (1984), Rajak *et al.* (1990), Hicks *et al.* (2001), and Aponte and Uribe (2001) as well as in field applications, *e.g.*, Kowalik and Stefaniak (1986); Siddaramaiah *et al.*(1986) and Chen *et al.* (1988).

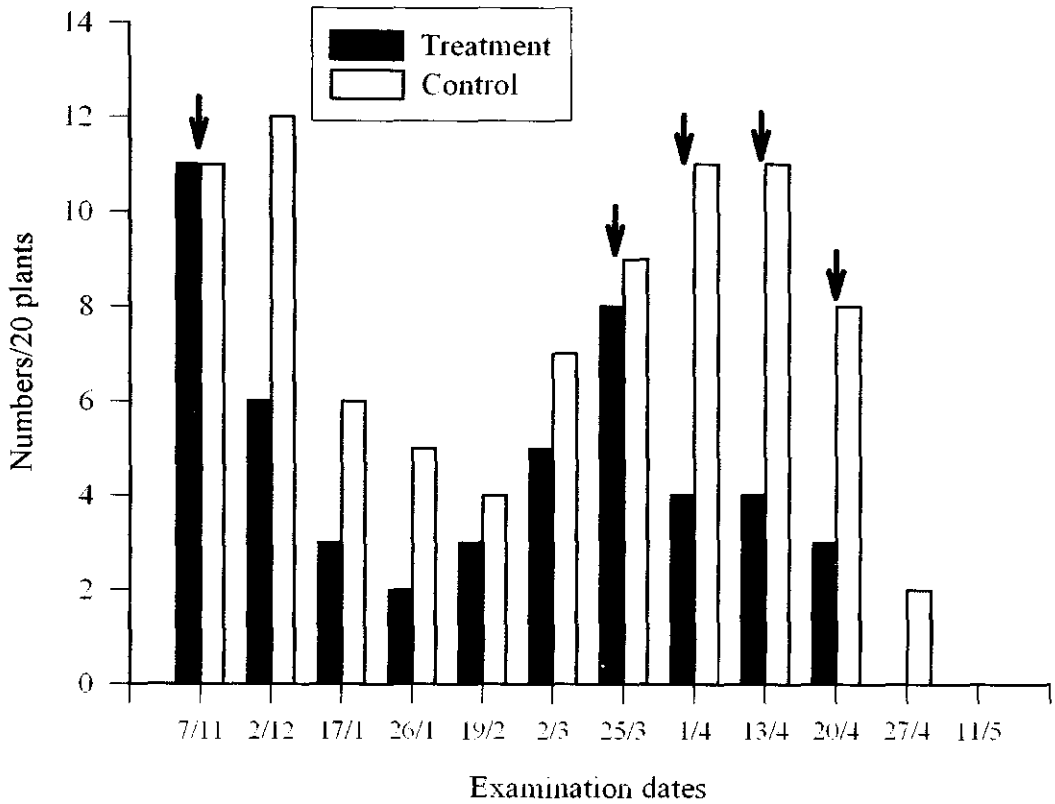

Fig. (1): Numbers of *Spodoptera littoralis* larvae after 5 sprays with fungi (arrows)

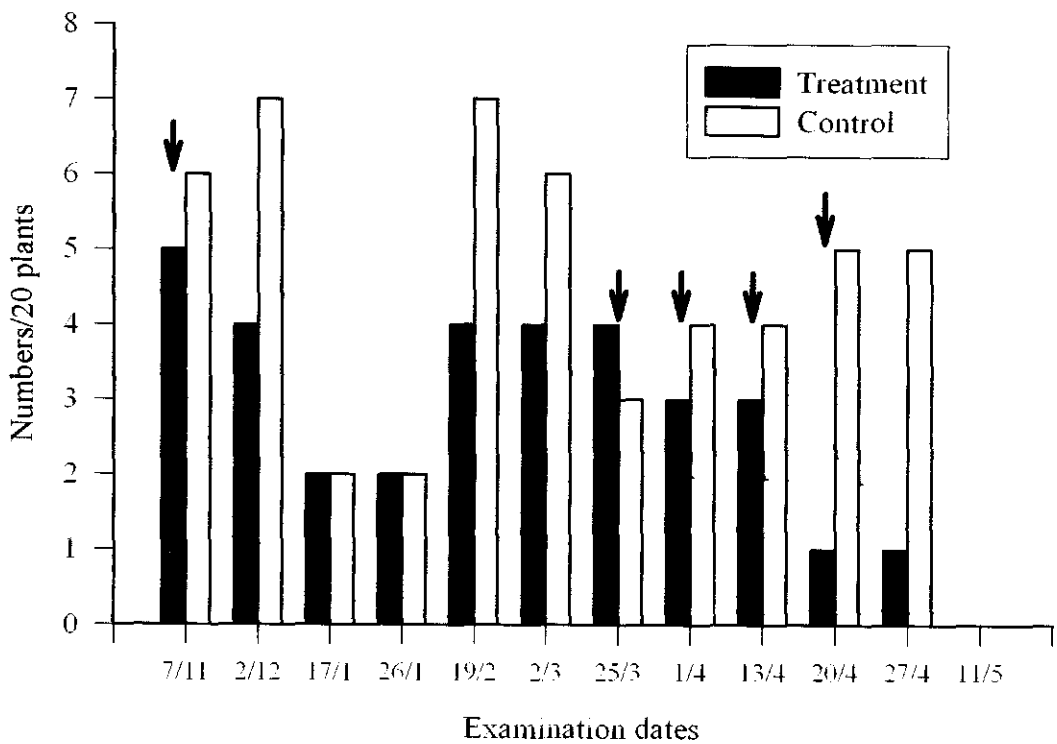

Fig. (2): Numbers of semi-looper larvae after 5 sprays with fungi (arrows)

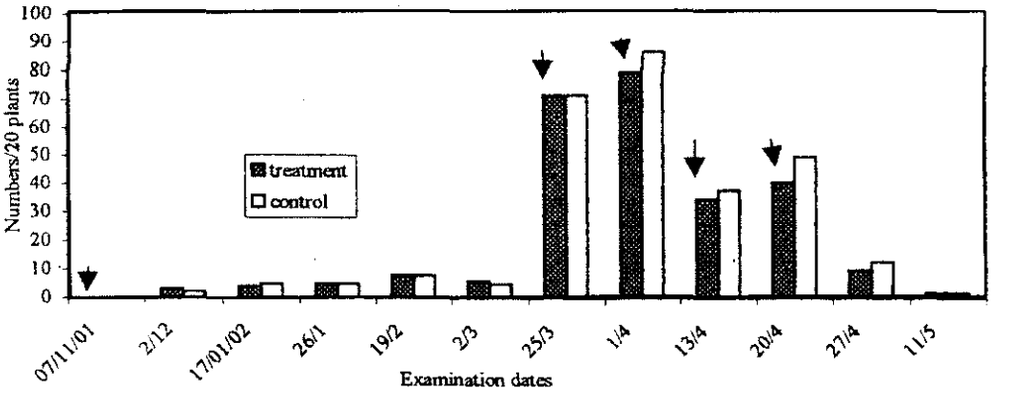

Fig.(3s) . Numbers of *Cassida vitata* eggs after 5 sprays with fungi

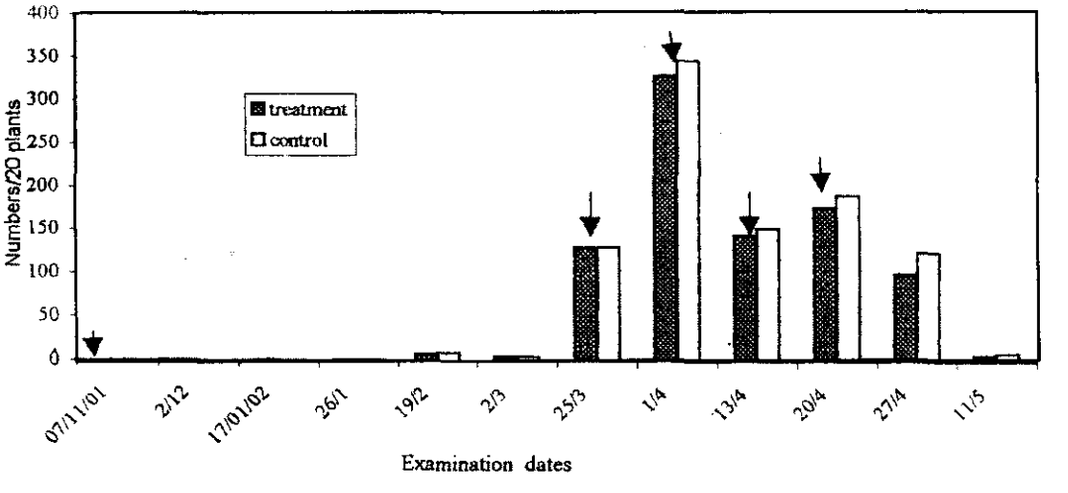

Fig.(3b) . Numbers of *Cassida vitata* larvae and pupae after 5 sprays (arrows) with fungi.

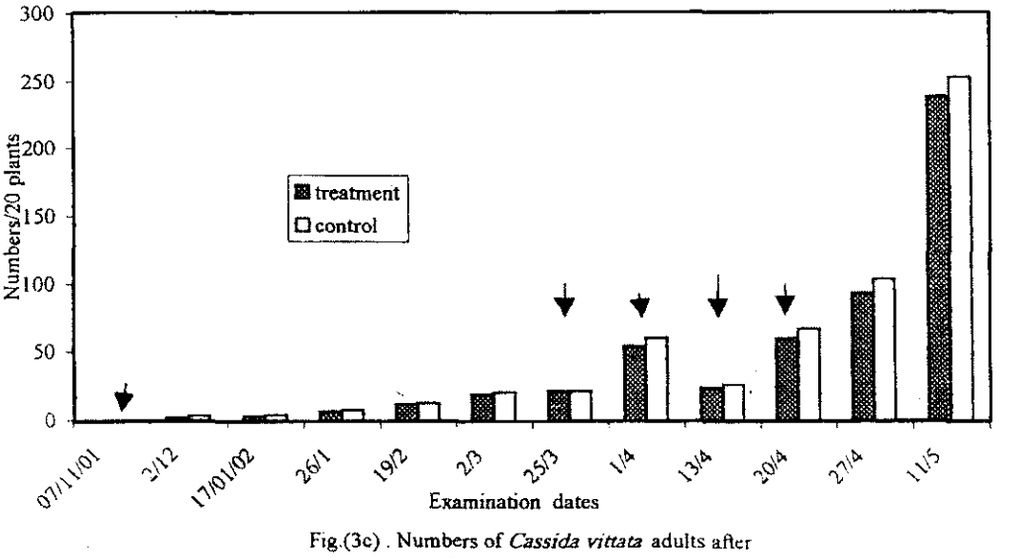

Fig.(3c) . Numbers of *Cassida vittata* adults after 5 sprays with fungi.

Table (3): Reduction% among noctuid larvae and the tortoise beetle after applications of the entomopathogenic fungi using the formula of Hendreson and Tilton (1955).

Date of field visits	*S. littoralis*	Semi-loopers	*C. vittata*		
			Eggs	Larvae & pupae	Adults
7/11/2001 *					
2/12/2001	50.0	42.8	0.0	0.0	0.0
25/3/2002 *					
1/4/2002 *	63.6	25.0	8.0	5.1	9.8
13/4/2002 **	63.0	25.0	7.8	4.6	7.4
20/4/2002 *	62.5	80.0	18.0	7.3	10.2
27/4/2002	100	80.0	4.1.0	20.0	9.5

* *Beauveria bassiana*, ***Metarhizium anisopliae*

The Semi-loopers:

They belong to more than one species, *i.e., Trichoplusia ni, Syngrapha circumflexa,* and *Autogapha gamma;* which are not differentiable from each other during the larval stage. They were considered here as one group of larvae: "semi-loopers". As show in Fig. (2) and Table (3), the *1st* spraying (7/11/2001) with *B. bassiana* resulted a control rate of 42.8%, 20 days after treatment among the young larvae (L_1-L_3). Meanwhile, the *2nd* application (25/3/2002) caused only 25% reduction among treated larval population that was more developed (L_3-L_5) than at the time of the *1st* application. This control rate (25%) remains also the same after the *3rd* application. But application of *M. anisopliae* at the same dose ($1x10^6$ conidia/feddan) by the *4th* spraying reduced the semi-looper larval population (L_3-L_5) by 80% one week after treatment; and remained at the same rate even after the *5th* application with *B. bassiana,* which may not be the reason for such reduction, as it remained the same counted figures at both treated and untreated plots (Fig2). Thus, *M. anisopliae* was found more successful in controlling larvae of the semi-loopers than *B. bassiana.* There is only one record (Kowalik and Stefaniak, 1986) referring to natural infection of *A. gamma* with *B. bassiana* and other fungi reaching 5.7% in cabbage and sugar beet. No other information is available on this subject in the literature, thus the present results could be considered as first record for successful control of semi-loopers with *B. bassiana.*

The Tortoise Beetle, *C. vittata*

The tortoise beetle, *C. vittata* became a major pest on the newly introduced sugar beet crop in Egypt (Salama and Elnagar, 1992; Ebieda *et al.,* 1996) that traditionally controlled by chemical insecticides (Saleh, 1994a&b; Ayala and Dominguez, 1996). It is believed that the intensive use of chemical insecticides in this crop had negatively suppressed the population of its natural enemies, especially its trichogrammatid egg parasitoid *Monorthochaeta nigra* Blood and Kryger reported by Awadalla (1996). No records for biological control measurements in sugar beet are available against this pest. Thus, the present results could be considered as first record for microbial control of *C. vittata.* As seen on Fig. (3a), the use of entomopathogenic fungi; *B. bassiana* and *M. anisopliae* sprayed in the field had resulted unsatisfactory control of the beetles eggs, because they were laid on the underside of the leaves, and accordingly they did not receive enough from the sprayed conidiospores. The reduction in eggs found on the treated sugar beet plants one week after the 2nd application with *B.bassiana* recorded only 8% that increased to 18% after the *4th* spraying with *M. anisopliae.* The *5th* spraying with *B. bassiana* (13/4/2002) induced only 4.1% reduction after one week.

Concerning the control of *C. vittata* larvae and pupae (Fig.3b and Table 3), the reduction rates recorded one week post the *2nd* and the *3rd* treatments with *B. bassiana* were 5.1 and 4.6%, followed by 7.3% after treatment with *M. anisopliae* that increased to 20% after an additional treatment (20/4/2002) with *B. bassiana.* Also, because of their presence on the underside of the leaves, the sprayed conidiospores did not reach them at this location as in the aforementioned case of the eggs.

Although adults of *C. vittata* are mobile than the larvae, but they prefer feeding on the underside of the leaves. Thus, it could be expected that the treatment with fungi using the spraying technique whatever the machine used, may explain the obtained low reduction rates among population of the adult beetles. Reduction rates of 9.8, 7.4, 10.2 and 9.5% were recorded one week after 4 successive applications with *B. bassiana* and *M. anisopliae* at the mentioned spraying dates (Fig. 3c and Table 3).

Although the laboratory bioassay of tested fungi proved highly infective to larvae, pupae and adults of *C. vittata* (El-Husseini, 2003), the application in the field was not successful. The main two reasons for this result are the location of the different insect stages on the underside of leaves; and that the spraying technique with the different spraying apparatus and motors whatever in low or high volume sprayings is not proper to reach the underside of the leaves. Thus, the dusting technique by which the applied material could be easily carried by air reaching the underside of leaves and accordingly contaminate the insects found there, might be the proper application technique for fungi conidiospores in the sugar beet crop simulating what is really happening in nature for infection with fungi spores. However, there is no available literature concerning the microbial control of the tortoise beetle with entomopathogenic fungi.

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