BIOLOGICAL AND HISTOLOGICAL EFFECTS OF *METARHIZIUM ANISOPLIAE* ON THE COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (BOISD.) (LEPIDOPTERA: NOCTUIDAE)

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Abstract

Toxicological, biological and histological effects of the isolated entomopathogenic fungus *Metarhizium anisopliae* from the red palm weevil adult on the newly molted 2nd and 4th instar larvae of the Egyptian cotton leaf worm, *Spodoptera littoralis* (Boisd.), were investigated under laboratory conditions at 27º ± 2º and 60% ± 5% R.H. Results revealed a decrease in the mean larval, pupal, and adult durations for the 2nd and 4th instar larvae surviving treatment with the LC_{50} of $2.8x10^8$ and $2.8x10^9$ spore/ml for the two tested instar larvae, respectively. Treatment also caused a reduction in the pupation and adult emergence percentage. There was also a great reduction in the mean number of eggs/female and the mean number of hatched eggs; The histological changes in the mid gut tissues in 6th instar surviving treatment with LC_{50} of *M. anisopliae* as newly ecdysed 3rd instar larvae was investigated**.** Sections in mid gut of untreated *S. littoralis* showed linning with columnar cells surrounded by cross longitudinal muscles fiber, the epithelial cells linning mid gut with its brush border and goblet cells. While, in mid gut of treated *S. littoralis* some epithelial cells which linning mid gut have lost their brush border, others were completely destructed.

Key words:
Spodoptera littoralis, Entomopathogenic fungi, *Metarhizium anisopliae,* Toxicology, Biology and Histology

INTRODUCTION

The Egyptian cotton leafworm, *S. littoralis* is an important pest in Egypt and other countries in Africa and Asia causing extensive economic losses in many cultivated crops (Frank *et al*., 1990). The extensive use of insecticides for controlling *S. littoralis* caused harmful effects on humans, living organisms and environment (Chaudhuri *et al*., 1999). The problems and hazards that have arisen as a result of using conventional insecticides were insentives for the search of alternative insecticides. Among these are microbial control agents which include bacteria, fungi, viruses, nematodes and protozoa (Dent, 2000). *Metarhizium anisopliae* (Metschnikoff) Sorokin is considered the most common entomopathogenic fungal species used as

biological control agents against insect pests (Barra *et al*., 2013). It is widely dispersed in nature and commonly isolated from infected insect or soil (Razinger *et al*., 2014).

The present study aimed to study toxicity of *M. anisopliae* as a new isolate as well as biological and histological effects of *S. littoralis* mid gut post treatment with *M. anisopliae*.

MATERIALS AND METHODS

Rearing of *S. littoralis*:

S. littoralis were provided by the Department of the Cotton Leafworm, Plant Protection Research Institute, Dokki-Giza. Newly hatched larvae were transferred to clean glass jars covered with muslin held in position with rubber bands and incubated under laboratory condition at 27º± 2ºC, 60 ± 5% RH, and 8:16 LD photoperiod. They were fed on castor leaves and examined daily. Upon pupation, pupae were collected; sexed and emerged moths were placed in pairs in glass jars supplied with leaves on Tafla, *Nerium oleander* (L.) as an oviposition site.

Isolated *M. anisopliae:*

The fungus used was isolated from adult of the red palm weevil, *Rhynchophorus ferrugineus* Oliv., in Insect Pathogen Production Unit, Plant Protection Research Institute and grown on a Sabouraud dextrose yeast agar (SDYA) culturing medium. The fungus was identified according to Barent and Hunter (1977).

Spores of isolated fungus cultured on Sabouraud dextrose yeast agar (SDYA) medium were harvested under sterile conditions using sterile distilled water containing 0.02% Tween-80®.

The number of spores were counted using Neubaur Hemocytometer to determine the concentration of spores/ml. Six concentrations were prepared from the stock suspension by serial dilution to be used in bioassay. They were $2.8x10^6$, $2.8x10^7$, $2.8x10^8$, $2.8x10^9$, $2.8x10^{10}$ and $2.8x10^{11}$ spores/ ml.

Two sets of five replicates were prepared each contains 10 newly molted 2nd and 4th instar larvae. The larvae were introduced into a cheese cloth and dipped into the prepared suspensions. The same numbers of larvae were considered as a control in which larvae were dipped in sterile distilled water containing 0.02% Tween-80®. All larvae were fed on clean and fresh castor oil leaves.

Mortality was recorded daily and accumulative larval mortality was determined at the end of the larval stage. The mortality percentages were corrected according to Abbott's formula (Abbott, 1925).

Toxicity was presented graphically as log/probit regression lines, LC_{25}, LC_{50}, LC_{75} and LC_{90} were estimated according to (Finney, 1971) using "LdPLine®" software.

Biological studies:

The 2nd and 4th larvae instar were collected and treated with LC_{50} value of *M. anisopliae* and the following biological parameters were studied: larval and pupal duration of each instar, and percentage of pupation. Pupae were sexed and then placed in pairs in the glass globes. Subsequently, the following was determined: percentage of adult emergence, longevity of moths and the fecundity and fertility of eggs/female. A control was set comprising a similar number of untreated moths.

Histopathological studies:

Samples of healthy and infected larvae (Third-instar larvae of *S. littoralis* infected with LC_{50} value of *M. anisopliae*) were fixed in 10% neutral formalin; the samples were dehydrated in graded ethanol to xylene and embedded in paraffin. Sections were cut at a thickness of 4-5µm and stained using Haematoxylin/eosin for larval tissue according to (Bancroft and Stevens, 1996). The stained sections were observed and photographed using an optical microscope.

RESULTS AND DISCUSSION

Bioassay:

As shown in (Table. 1 and Fig. 1) the efficacy of *M. anisopliae* conidia on the 2nd and 4th instar larvae of *S. littoralis* was expressed in terms of LC_{25}, LC_{50}, LC_{75} and LC_{90}. It was found that this isolate is pathogenic to the two tested instar larvae. The probit analysis indicated that LC_{25}, LC_{50}, LC_{75} and LC_{90} values of 2nd and 4th instar larvae after 48 hr. of treatment were 2.8×10^6 and $2.8x10^7$, $2.8x10^8$ and $2.8x10^9$, $2.8x10^{10}$ and $2.8x10^{11}$ and $2.8x10^{12}$ and $2.8x10^{12}$ spores/ml. respectively,. These results agree with those of Loc *et al.* (2010) who studied the effect of *B. bassiana and M. anisopliae* on Black citrus aphids and *Citrus pyrilla*. In addition, when De Senna-Nunes *et al.* (2002) evaluated the effects of two isolates of *A. flavus* and two isolates of *Penicillium corylophilum* on adults of *Musca domestica*, they found that one of the *A. flavus* isolates killed 100% of flies three days after treatment, while the two *P. corylophilum* isolates killed 100% of flies on the 7th day after treatment.

M. anisopliae is one of several natural agents for controlling a broad range of insects by direct penetration of the host cuticle. (Tin *et al.*, 2008). There is an increasing interest in the use of entomopathogenic fungi for the biocontrol of insect pests (Evans, 1999).

The significance of the present study is to illustrate the ability of the tested entomopathogenic fungal isolate**;** *M. anisopliae* to show positive influences on larval mortality as well as induced malformation in treated 2nd and 4th instar larvae of *S. littoralis*. Spores were used in different concentrations to estimate efficiency of the fungal isolate as a biological control against 2nd and 4th instar larvae of *S. littoralis* under laboratory conditions.

Table 1. Susceptibility of S. littoralis 2nd and 4th instars larvae to M. anisopliae

Instars	LC_{25} (Spores/ml)	LC_{50} (Spores/ml)	LC_{75} (Spores/ml)	LC_{90} (Spores/ml)	Slope
2nd instars	$2.8x10^{6}$	$2.8x10^{8}$	$2.8x10^{10}$	$2.8x10^{12}$	0.3283
4th instars	$2.8x10^{7}$	$2.8x10^{9}$	$2.8x10^{11}$	$2.8x10^{12}$	0.3189

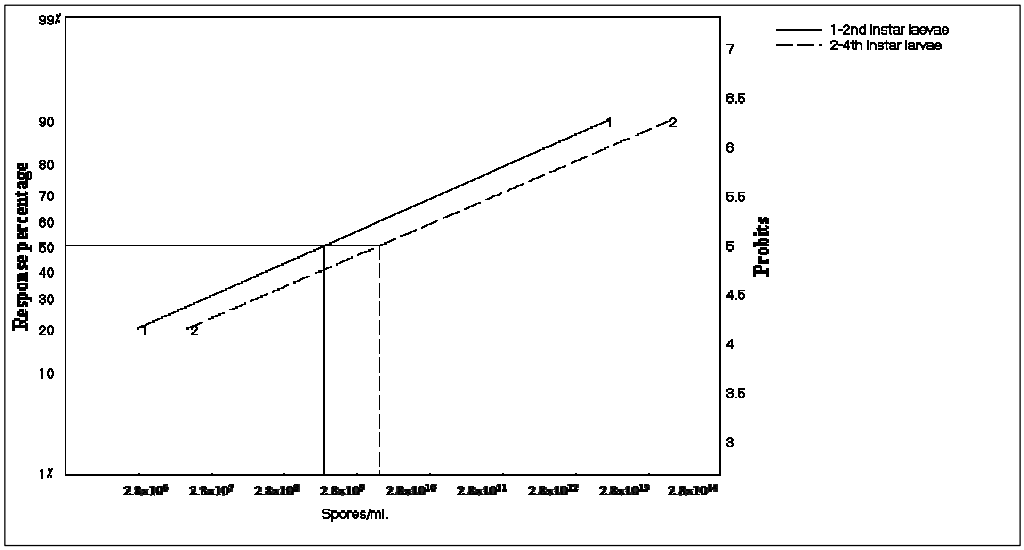

Fig. 1. Concentration log probit toxicity lines of *M. anisopliae* against 2nd and 4nd *S.littoralis*.

Effect on biological aspects:

The results in Table (2) summarized the duration of larvae treated as 2nd instars lasted 12 days up to pupation which was less than the control by nearly four days. Meanwhile, pupal stage durated 12.3 days as compared to 13 days in the control, *i.e*. nearly a day less. Treatment of 4th instars larvae with LC_{50} of *M. anisopliae* also increased the remaining larval instars duration to 11 days as compared to 12 days in the control. Meanwhile, pupal duration was 11.3 days as compared to 13 days in the control, These differences were found to be statistically significant.

The percentage of larvae entering pupation was markedly less in treated 2nd and 4th instars larvae which were 49.2 and 50 %, respectively, less than the control. Meanwhile, percentage of adult emergence was slightly decreased than the control to 92 and 95% for the respective mentioned treated instars. Moth's emerging from treated 2nd or 4th instar larvae with LC_{50} of *M. anisopliae* showed a shorter life span than the untreated insects.

Table (3) shows that the mean number of eggs laid for both females emerged from treated *S. littoralis* 2nd and 4th instar larvae was decreased significantly, compared with their counterpart of the untreated ones. This was also clear in the mean number of hatched eggs compared to the controls. The number of eggs laid was recorded in the mating treated moths resulted from treated 2nd and 4th instar larvae giving a mean of 847 and 671 eggs, respectively. The number of hatched eggs was recorded for the mating combination of treated moths resulted from treated 2nd instar larvae giving a mean of 528 hatched eggs while those from treated 4th instar larvae gave a mean of 622 hatched eggs compared to the normal moths that gave a mean number of 2103 hatched when resulted from treated 2nd instar larvae and 1827 hatched eggs for the 4th instar. These differences were found to be statistically very highly significant.

These results agreed with those of Hafez *et al*. (1997) who treated *Phthorimaea operculella* larvae with *Beauveria bassiana*; and Noma and Strickler (2000) who investigated the effects of *B. bassiana* infection on ovipositional behavior of *Lygus hesperus*. In addition, when Dubois *et al*. (2004) investigated the effect of two commercial preparations of *B. bassiana* and *B. brongniartii*, they found a reduction in adult longevity of the beetle *Anoplophora glabripennis*. Also, the obtained results agreed with those of Abd El-Kareem (2007) who treated larvae of *Ostrinia nubilalis* with *A. flavus*.

Table 2. Effect of LC_{50} of *M. anisopliae* on the mean larval duration, pupation percentage, mean pupal duration, adult emergence and mean adult longevity of *S. littoralis* treated as 2nd and 4th instars larvae.

Treated Instars	Mean larval duration (days± S.E.)	Pupation %	Mean pupal stage (days± S.E.)	Adult emergence %	Mean adult longevity (days± S.E.)	
					♀	♂
2nd instars	12.5±0.28**	49.2	12± 0.1*	92	11.3± 0.30*	12.33±0.14**
Control	16± 0.28	100	13± 0.5	100	12.67± 0.38	14.33± 0.11
4th instars	11± 0.3*	50	11.3± 0.1*	95	11.33± 0.1**	13.67±0.28**
Control	12± 0.1	100	13± 0.1	100	12.33± 0.1	15± 0.38

*: Significant at P< 0.05 **: highly significant a P< 0.01

Table 3. Effect of LC50 of M. anisopliae on reproductive potential of S. littoralis treated as 2nd and 4th instars larvae.

Mating Combination	No. of eggs/♀ mean±S.E.		No. of hatched eggs (Mean±S.E.)	
	2nd	4th	2nd	4th
Treated	847±16.8***	671±18.4***	528±8.5***	622±4.04***
Control	2135±60.6	1875±15.1	2103±4.04	1827±12.11

***: Very highly significant a P< 0.001.

Histopathological effects:

The histological structure of mid gut in larvae of Lepidoptera is well documented (Chapman, 1988). As seen in Fig. (2), the mid gut is lined with an epithelial layer, which rests on a basement membrane, and is composed of a single layer of three types of cells. (i) A majority of columnar cells containing a large coarse nucleus which occupies a middle position within the cell and bears a striated or brush-like border (microvilli). (ii) Goblet cells; which are somewhat calyx-shaped and are seen between the columnar cells; each of these cells has a large ampulla opening by a narrow neck through a small aperture on the inner surface. (iii) Regenerative cells are small in size and rest on the basement membrane between the bases of the other cells, and are round or elongated and contains a large nucleus surrounded by a small amount of strongly basophilic cytoplasm.

Within the midgut lumen, there is a thin peritrophic membrane, that surrounds the food mass. A musculosa surrounds the epithelial layer, composed of an

inner circular layer and an outer layer of longitudinal muscle. As seen as in Fig. (3) The histological structure of the midgut of treated larvae many histological change than that the control. The peritrophic membrane was not closely lying to epithelial cells and the space in between the epithelium and peritrophic membrane was filled with few cytoplasmic vesicles. The obtained results agreed with those of Quesada-Morga *et al.* (2006) who found that crude soluble protein extracted from *M. anisopliae* elicited many histological changes to the midgut of 2nd instar *S. littoralis* larvae: the midgut epithelium showed deterioration, there was destruction of the microvilli of the columnar cells, and formation of vacuoles. The histopathological changes induced by *M. anisopliae* treatment was more or less similar to histological changes detected by Thorvilson *et al.* (1985) and Srisukchayakul *et al.* (2005). Lysis of the cuticlar layer occurred before hyphae penetrated and invaded the epidermis. In addition, the epithelial cells of the midgut were highly affected because of fungal infection.

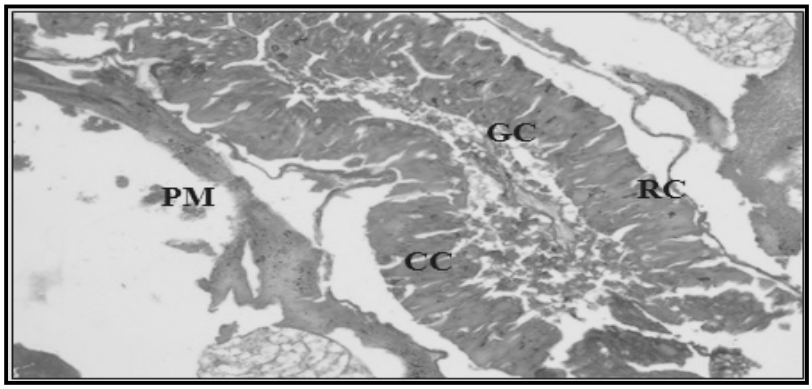

Fig. 2. Midgut of *S. littoralis* showing normal structure of:
PM : Peritrophic membrane, CC : Columnar cells,
GC : Goblet cells, RC : Regenerative cells.

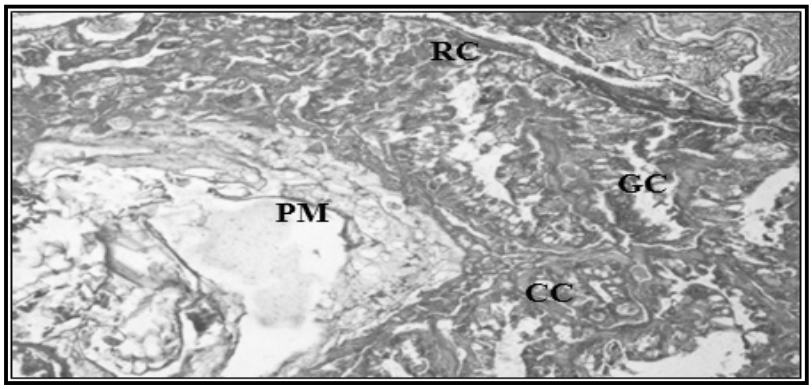

Fig. 3. Severe proliferation of some columnar cells as well as goblet cells other cells were necrosed.
PM : Peritrophic membrane, CC : Columnar cells,
GC : Goblet cells, RC : Regenerative cells.

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التأثيرات البيولوجية والهيستولوجية لفطر *Metarhizium anisopliae* على دودة ورق القطن الكبرى

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تمت دراسة التأثير السمي والبيولوجي و الهيستولوجي للفطر الممرض للحشرات *Metarhizium anisopliae* المعزول من الحشرة الكاملة لسوسة النخيل الحمراء،على يرقات كل من العمريين الثاني والرابع لدودة ورق القطن الكبرى تحت الظروف المعملية على درجة حرارة ٢٧ ±٢ م° ، ورطوبة نسبيه ٦٠% ±٥ %. أدت المعاملة بصفة عامة بالتركيز نصف المميت إلى خفض في متوسط العمر اليرقي والعذري وانخفاض في نسبه التعذير ونسبه خروج الفراشات مقارنه باليرقات غير المعاملة، كما أدت أيضا إلى خفض متوسط عمر الطور البالغ مقارنه بالكنترول. أدت المعاملة أيضا إلى خفض الكفاءة التناسلية للفراشات الناتجة من اليرقات المعاملة بالفطر محل الدراسة حيث ظهر ذلك في انخفاض متوسط عدد البيض ومتوسط الفقس الناتج مقارنة بالكنترول. وتم عمل قطاعات في المعي الاوسط حيث وجد تدمير كامل وخلل في جميع انسجه وخلايا المعي الاوسط للحشرة المعاملة مقارنه بالحشرات غير المعاملة.

الكلمات المفتاحيه:

دودة ورق القطن، الفطر الممرض للحشرات *Metarhizium anisopliae* ، الهيستوباثولوجي.