EFFECT OF SECONDARY METABOLITES FROM THREE ENTOMOPATHOGENIC FUNGI ON THE COTTON LEAF WORM, SPODOPTERA LITTORALIS (BOISD.)

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Abstract

Three entomopathogenic fungi isolates, *i. e., Beauveria bassiana, Paecilomyces fumosoroseus* and *Metarhizium anisopliae* were used as biological control agents against the Egyptain cotton leaf worm, *Spodoptera littoralis* (Boisd.), which represent the most severe destructive cotton pest in Egypt and many other countries.

The entomopathogenic fungi were inoculated YES medium, incubation period was carried out for 19 days at pH 5.4 and 28 – 30ºC. After incubation period, the mats of the fungi were removed and YES broth was mixed individually with chloroform/methanol (2:1, v/v). The mixture was shaken vigorously in a separating funnel and left to settle down forming a dense lower aqueous layer containing the secondary metabolites. Extracted metabolites were then concentrated by using a speed vacuum device (Maxi Dry Plus) to a volume of 1 ml.

Virulence of *B. bassiana, M. anisopliae* and *P. fumosoroseus* was investigated against 1st instar larvae of the cotton leaf worm *S. littoralis*. Results obtained revealed that the three metabolites (toxins) were toxic to the 1st instar larvae of *S. littoralis*. The most effective toxin was *M. anisopliae* (84.92 %) followed by *P. fumosoroseus* (66.32%) and *B. bassiana* (62.65 %).

On the other hand, LT_{50} values in days for *M. anisopliae* were 8.5414, 6.4808, 4.7721, 3.7211, while for *P. fumosoroseus* they were 11.0488, 7.7757, 6.4998, 5.1703 and for *B. bassiana* they were 10.4380, 8.3415, 6.8299, 5.6922 in relation to the concentrations 25, 50, 75, 100% respectively.

Treatment of *S. littoralis* 4th instars with LC_{50} of the metabolic fungal toxins caused complete disruption of endocuticle of the resulting late 6th instars. Meanwhile, it caused exfoliation of the mid gut epithelium from the underlying circular muscle fibers, leaving a large vacuole or space, disruption of both the peritrophic membrane and columnar cells, beside high vacuolization and collapsing of the fat body.

INTRODUCTION

Cotton crop is one of the major sources for the economy in A.R.E. and other countries. The Egyptian cotton leaf worm *Spodoptera littoralis* (Boisduval) is one of the most important pests in Egypt, eastern Mediterranean region and other countries

in Africa and Asia. Several plant hosts specially the cotton crop were attacked by the cotton leaf worm, during the growing season and causes great damage to a wide variety of crops including vegetables and fruit trees.

Until present, the hand picking of egg-masses is used as first step in the cotton leaf worm control, supplemented by the use of chemical insecticides. The problems followed the use of insecticides in control pest derived the attention to find alternative safe methods for cotton leaf worm control.

Insecticide resistance and the demand for reduced chemical inputs in agriculture led to the development of alternative forms of pest control. Biological means offer an attractive or supplement to the use of chemical pesticides. Microbial biocontrol agents are naturally occurring organisms and perceived as being less damaging to their environment. Furthermore, their generally complex mode of action makes it unlikely that resistance could be developed to bio-pesticides. The use of microorganisms as selective pesticides has had some notable successes. Great efforts have been made to control this pest chemically. Due to the continuous use of chemical pesticides against this pest, resistance to the action of pesticides had dramatically evolved. Also, the extensive use of these chemicals has given rise to problems such as residual toxicity (pollution) and harmful efforts on beneficial insects, which are natural enemies of target or non-target pest species. Such problems have become a cause of search for more safe pesticides including microbial agent as fungi, bacteria and viruses **(**Rashed 1993).

Generally, the death of on insect results from a combination of factors such as mechanical damage which resulting from tissue invasion, depletion of nutrient resources and toxicosis. Other factors, which may have a role in the demise of the insect hosts, are a range of low molecular weight insecticidal toxins produced by entomopathogenic fungi, e. *g.*, Beauvericin, Beauverolides, Bassianolide and Isarolides which are produced by *Beauveria bssiana* (Elsworth 1977**)** as well as Destruxins and Cytochalasins produced by *Metarhizium anisopliae.*

MATERIALS AND METHODS

Extraction of secondary metabolites:

The fungi were inoculated to YES medium, incubation was carried out for19 days at pH 5.4 and incubated at 28–30ºC. After incubation, the mats were removed and YES broth was mixed individually with chloroform/methanol (2:1v/v).The mixture was shaken vigorously and left to settle down forming a dense lower aqueous layer

containing the secondary metabolites. Extracted metabolites were then concentrated using a speed vacuum device (Maxi Dry Plus) to 1 ml.

Toxin production medium

Yeast Extract Sucrose liquid medium g/l:Sucrose, 150 and yeast extract , 20.

Test insect

The larvae were maintained on the semi-synthetic diet as described by (Mabrouk, *et al.*, 1996).

Toxocological studies

S. littoralis larvae were obtained from the laboratory culture at Plant Protection Research Institute. Newly hatched larvae from a single egg batch were introduced to pots containing 0.5 cm thick the synthetic diet using a soft hairbrush, then transferred to plastic "poly pots" with dimensions of 5cm deep and 10cm internal diameter. Ten larvae were reared in each plastic cup until reaching pupae. Freshly emerged moths were coupled in small glass jars provided with a filter paper as a site for egg- laying, and supplied with 10% sugar or honey solution soaked in a peace of cotton wool. All experiments were carried out under constant conditions of 26±2ºC and 60±5 RH.

Virulence of isolated entomopathogenic fungi:

Newly hatched larvae from a single egg batch was introduced to pots containing treated synthetic diet, three compounds were used. Four concentrations of each compound were prepared 100%, 75%, 50% & 25%. 500 µl from each dilution were added to 5 gm of diet. The larvae were left to feed on treated diet for 48 hrs, and then mortality percentages were recorded. The survived larvae were transferred to feed on untreated diet. Mortality percentages were corrected using Abbott's formula (Abbott 1925). The L_{C50} and L_{C90} values were calculated according to method of (Finney 1971).

Histopathological effects of the fungal toxins on integument, fat body and midgut:

The concentration of LC_{50} of *M. anisopliae* toxins was applied in the treatment. The influences of the entomopathogenic fungus *M. anisopliae* on the integument, fat bodies as well as mid-gut were studied.

After feeding the 4^{th} instar larvae on treated diet for 48 hrs, they were transferred onto untreated diet. The survived larvae were collected after 96 hrs.

In each treatment, five larvae were selected and starved for 4 hrs. to insure that the mid-gut is as empty as possible, then dissected by using the binocular microscope in Ringer's saline solution (0.0065%). The mid-gut, chitin and fat bodies were removed and fixed in alcoholic Bouin's fluid for 24 hrs., washed several times in 70% ethyl alcohol to remove most of the fixative and then dehydration takes place through

ascending series of ethyl alcohol, 70, 80, 90, and 95% followed by two changes of absolute ethyl alcohol (30 min. for each) then transferred to a mixture of absolute ethyl alcohol and xylene and then to xylene (30 min. for each). After that, infiltration with wax took place by transferring to xylene-paraffin mixture for 30 min. and then to three successive paraffin baths (30 min. for each) at 58° - 60°C, then embedded in pure paraffin. Sectioning was done by a Rotary microtome at thickness of 5 ц. Paraffin ribbons were affixed on clean glass microscopal slides and flattened on hot plate for a period of 15 min., then stained by using double stain, *i.e.*, haematoxylin for nucleus and eosin for cytoplasm and cell wall. The staining procedure took place as follow: dewaxation carried out by passing the slides through xylene I, xylene П and then to a mixture of xylene and absolute ethyl alcohol (5 min. for each) and then hydrate to water by running through descending series (100, 95, 90, 80, 70, 50, and 30%) of ethyl alcohol and then to distilled water (2 min. for each). Sections were stained in haematoxylin for 5 min. and rinsed in distilled water. Destained in 70% acid alcohol until light pink colour of specimen appeared and then neutralized in tap water, stained with eosin (15 sec.) and washed in 2 changes of 95% ethyl alcohol then in absolute ethyl alcohol (5 min. for each). Clearing occured in 2 changes of xylene (5 min. for each), and then mounted with Canda balsam, covered with clean glass covers and left to dry in an electric oven at 37°C. To evaluate the histopathological effects on the adult stage, virgin adults that resulted from treated newly hatched larvae were also dissected in Ringer's saline solution to examine testis and ovaries. Testis undergo the same previous technique, however, as for ovaries, they differ in certain steps in which, after fixation and dehydration, they transferred from 95% ethyl alcohol to methyl benzoate for clearing until it settled in the bottles bottom, washed with benzene for 5 min., then transferred to benzene and wax mixture and then running to three bathes of paraffin wax (30 min. for each), and then followed the routine technique previously described for larvae.

RESULTS AND DISCUSSION

Virulence of isolated entomopathogenic fungi.

Virulence of the fungal species under study was investigated against 1st instars larvae of cotton leaf worm S. littoralis as shown in Tables 1, 2, 3 and figures 1,2,3. Results obtained revealed that the three tested metabolites were toxic to the 1st instars larvae of S. littoralis. The most effective was that of M. anisopliae followed by B. bassiana and P. fumosoroseus. The mortality percentages after 8 days were 39.46, 48.51, 55.44 & 62.65% for the concentrations 25, 50, 75, 100% of B. bassiana, 47.36,58.58,

73.04 & 84.92% for the same concentrations of M. anisopliae and 38.26, 51.25, 58.94 & 66.32% for P. fumosoroseus. Data in tables 4,5,6 represents LT_{50} values in days for B. bassiana to concentrations 25, 50, 75, 100%.

Table 1. Corrected cumulative mortality percentages of 1st instars larvae of *Spodoptera littoralis* after feeding on synthetic diet treated with metabolic toxins of *Beauveria bassiana*.

Conc. (%)	Cumulative mortality % at indicated days after treatment			
	2	4	6	8
25	4.84	16.76	28.90	39.46
50	10.05	25.52	38.40	48.51
75	14.39	32.17	45.54	55.44
100	16.08	36.91	51.99	62.65

Table 2. Corrected cumulative mortality percentages of 1st instar larvae of *Spodoptera littoralis* after feeding on synthetic diet treated with metabolic toxins of *Metarhizium anisopliae*.

Conc. (%)	*Cumulative mortality % of indicated days after treatment*			
	2	4	6	8
25	7.08	22.12	36.03	47.36
50	11.32	30.98	46.84	58.58
75	15.07	41.69	60.72	73.04
100	20.38	54.08	74.11	84.92

Table 3. Corrected cumulative mortality percentages of 1st instars larvae of *Spodoptera littoralis* after feeding on synthetic diet treated with metabolic toxins of *Paecilomyces fumosoroseus*.

Conc. (%)	Cumulative mortality % of indicated days after treatment			
	2	4	6	8
25	5.69	17.36	28.61	38.26
50	6.77	23.24	38.78	51.25
75	9.97	29.85	46.53	58.94
100	17.97	40.22	55.71	66.32

Table 4. LT_{50} fucidal limits and slope values of 1st instars larvae of *Spodoptera littoralis* after feeding on treated synthetic diet with metabolic toxins of *Beauveria bassiana.*

Conc.	LT_{50} (days)	95% Fucidal limits		A Intercept	b Slope
		Lower	Upper		
25	10.4380	7.7256	23.1728	2.6435 ±0.4538	2.3135 ±0.6086
50	8.3415	6.3753	15.5157	3.1005±0.3819	2.619±0.5277
75	6.8299	5.3521	10.9044	3.3370 ±0.3539	1.9930 ±0.4973
100	5.6922	4.5871	7.7397	3.3519 ±0.3466	2.1821 ±0.4911

Table 5. LT_{50} fucidal limits and slope values of 1st instar larvae of *Spodoptera littoralis* after feeding on treated synthetic diet with metabolic toxins of *Metarhizium anisopliae*

Conc. (%)	LT_{50} (days)	95% Fucidal limits		A Intercept	b Slope
		Lower	Upper		
25	8.5414	6.6590	14.7417	2.8284 ± 0.4128	2.3312 ±0.5621
50	6.4808	5.2842	8.4186	3.0775 ±0.2732	2.3687 ±0.3659
75	4.7721	3.9702	5.7777	3.1427 ±0.3530	2.7365 ±0.5040
100	3.7211	3.1065	4.3460	3.2413 ±0.2530	3.0912 ±0.3577

Table 6. LT_{50} fucidal limits and slope values of 1st instar larvae of *Spodoptera littoralis* after feeding on treated synthetic diet with metabolic toxins of *Paecilomyces fumosoroseus*

Conc. (%)	LT_{50} (days)	95% Fiducial limits		A Intercept	B Slope
		Lower	Upper		
25	11.0488	7.9041	29.1485	2.7774	2.1303 ±0.5909
50	7.7757	6.2653	11.7734	2.7441	2.5326 ±0.5660
75	6.4998	5.3439	8.8618	2.9619	2.5072 ±0.5288
100	5.1703	4.1655	6.7334	3.4148	2.2217 ±0.4854

Table 7. Values of LC_{25}, LC_{50} of metabolic toxins against 1st instar larvae of *Spodoptera littoralis* (Boisd)

Fungi (Pathogens)	LC_{25} values	LC_{50} values (%)
Metarhizium anisoplae	11.631	27.538
Beauveria bassiana	11.867	53.478
Paecilomyces fumosoroseus	15.759	48.448

Data in table 7 showed lethal concentrations required to kill 25 % and 50 % of treated larvae of *S. littoralis*, where LC_{25} was 11.631%, 11.867% and 15.759 % for *M. anisopliae, B. bassiana* & *P. fumosoroseus*, respectively. LC_{50} recorded 27.538 % for *M. anisopliae*, 53.478 for *B. bassiana* and 48.448 % for *P. fumosoroseus*. All tested isolates were found to be virulent to the test insect. The obtained results showed that *M. anisopliae* was the most effective isolate (84.92 %) then *P. fumosoroseus* (66.32 %) and *B. bassiana* (62.65 %) after 8 days. Present data reveal differences in virulence among the 3-entomopathogenic fungi isolates. This isolates specific differences in virulence exemplified the interspecific variation exists in entomopathogenic fungi even when insect hosts were the same bioassay species. This is agree with (Hsieh *et al*., 1998) as they found that the extract of fermentation broth of *M. anisopliae var. anisopliae* showed high virulence to *S. exigua* as 86.7 % mortality of 3rd instars larvae after 24 hours of infection and 95 % mortality after 2 days.

(St. Leger 1995) mentioned that there are several broad classes of pathogenicity genes. Other pathogenicity genes may encode enzymes that allow the fungus to overcome their host barriers. Therefore extensive genetic variation in pathogenicity waits characterization at the molecular level

Histological studies

The integument of normal 6th instars of *S. littoralis* consists of cuticle, hypodermis and basement membrane. Fig. (1), the cuticle is divided into three layers, the epicuticle followed by the exocuticle and endocuticle. The hypodermis consists of a single layer of less cuboidal or columnar cells. Each cell contains a large nucleus. The basement membrane is attached to the basal surface of the hypodermal cells. Treatment of 4th instars with LC_{50} of the metabolic fungal toxins completely disrupt the formation of endocuticle of the resulting late 6th instars. Fig. (2).

Mid gut

It is composed of two layers of muscle fibers. Next to the inner layer inwards is a basement membrane, followed by an epithelial layers of cells lining the mid gut cavity within the lumen, there is a thin peritrophic membrane. Fig. (3).

Microscopic examination revealed that treatment of 4th instars with LC_{50} of the metabolic fungal toxins caused exfoliation of the mid gut epithelium from the underlying circular muscle fibers, leaving a large vacuole, disruption of both the peritrophic membrane and columnar cells. Fig (4).

Fat body

The fat body of normal 6th instars of *S. littoralis* in section is ribbon-like. The fat body cells are closely adherent and a delicate membranous sheath covers the external surface of the cell masses. Fig. (5).Treatment of 4th instars caused high vacuolization and collapsing of the fat body. Fig. (6).

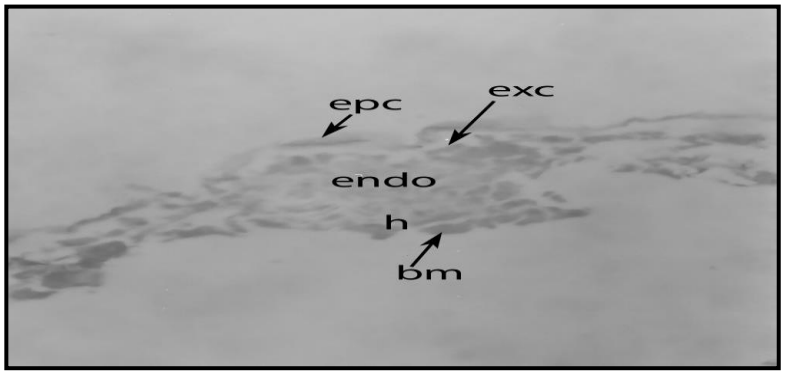

Fig. 1. Photomicrograph of L.S. in the integument of normal late 6th larval instar of *Spodoptera littoralis* where Bm=Basement membrane ,end.=endocuticle , Epc.=Epicuticle , Exc.=exocuticle , h.=Hypodermis

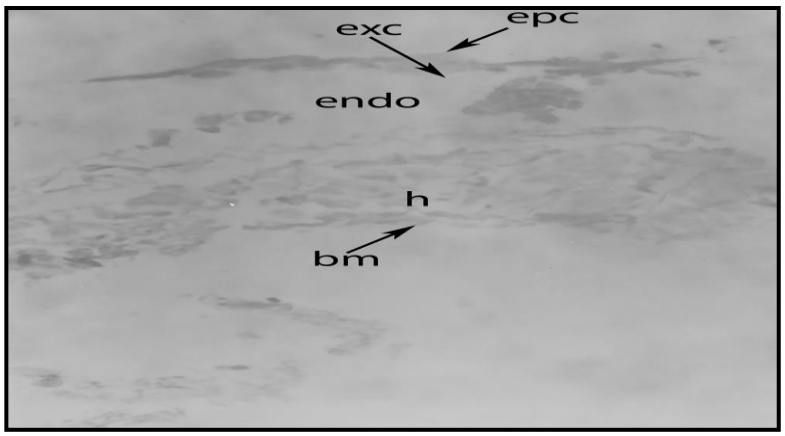

Fig. 2. Photomicrograph of LS. in the integument of late 6th larval instar of S littoralis after feeding on synthetic diet treated with LC50 of metabolic toxins of Metarhizium anisopliae.

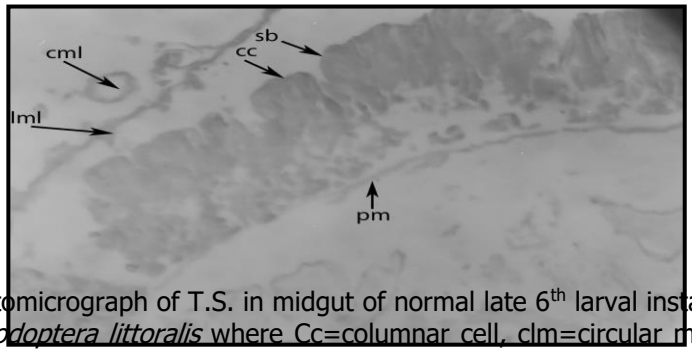

Fig. 3. Photomicrograph of T.S. in midgut of normal late 6th larval instars of *Spodoptera littoralis* where Cc=columnar cell, clm=circular muscle layer , Ge=globlet cell ,lml=longitudinal muscle layer , Pm=Peritrophic membrane, re=regenerative cell , Sh.=striated border , v.= vacuole

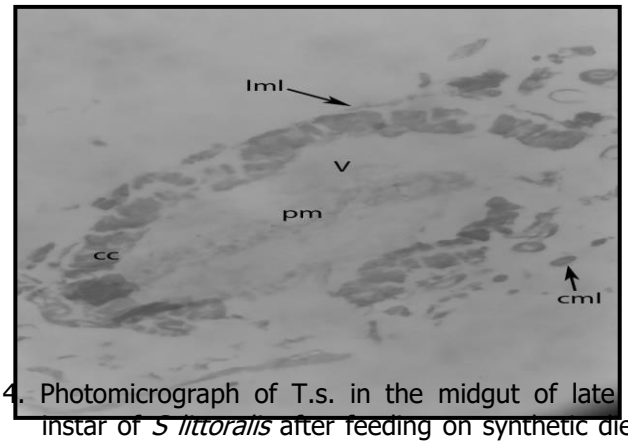

Fig. 4. Photomicrograph of T.s. in the midgut of late 6th larval instar of *S littoralis* after feeding on synthetic diet treated with LC_{50} of metabolic toxins of *Metarhizium anisopliae*.

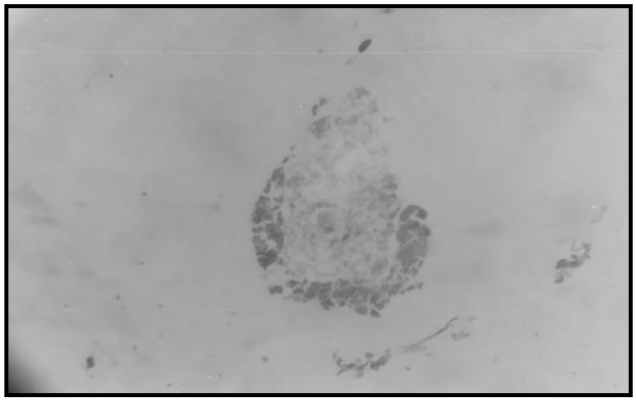

Fig. 5. Photomicrograph of L.s. in the fat body of normal late 6th larval instar of *S littoralis*.

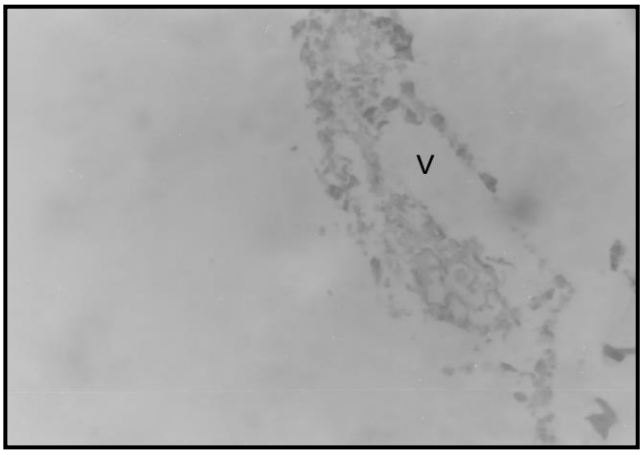

Fig. 6. Photomicrograph of L.s. in the fat body of late 6th larval instar of *S littoralis* after feeding on synthetic diet treated with LC_{50} of metabolic toxins of *Metarhizium anisopliae*. v: vacuole

Histopathological changes due to the effect of various toxicants have been a subject of considerable discussion among authors as the primary cause of insect's inactivity and consequent death. Many of the histopathological changes of the cuticle observed in the present study for *S. littoralis* late 6th instars due to treatment of 4th instar ones with entompathogenic fungal toxins. The obtained results agreed with (Hegazy, 1990) when using different chitin synthesis inhibitors against the same insect species. (Philogene and McFarlane 1967) established quite thoroughly the evidence necessary to conclude that the process of chitin synthesis is extracellular. They reported that cuticle precursor forms layers on the surface or very close to the surface of secretory cells, and that cuticular deposition may be completed at a distance from the secretory sites. They also linked oenocytoids and their secretions with cuticle formation in vivo. Moreover, (Essawy *et. al.*, 1985) observed phenoloxidase activity in this type of cells.

The collapsing, lysis and vacuolization of the fat body of *S. littoralis* late 6th instars appeared in treatment with entomopathogenic fungal toxins. Results obtained with 4th instars agree with those of (Wang and Zhang 1987) for the fat body of *Pieris rape* larvae treated with an unspecified chitin synthesis inhibitor (designated as No. 3), and by (Meola *et al.*, 1996) for the fat body of *Ctenocephalidis felis* adults treated with pyriproxyfen. (Munson 1954) reported that fat body of insect plays an important role in the storage of insecticides.

Many of the histopathological alterations reported in the present study for the midgut of *S. littoralis* late 6th instars treated with the three entomopathogenic fungi as 4th instars agree with those reported by other authors. (AboEl-Ghar *et. al.*, 1994) reported that 0.01 ppm of diflubenzuron caused vacuolization of the midgut epithelium of *S. littoralis* larvae, in addition to the

sloughing off of scattered groups of the midgut epithelium into the gut lumen and disappearance of the cell boundaries. Similar observations were also recorded for the midgut epithelium of the chironomid larvae, *Chironomus decorus* and *Tapnypus grodhausi*, when each was treated with both of diflubenzuron and triflumuron (Pelsue 1985), the cat flea adult, *Ctenocephalidis felis* (Meola *et al*., 1996) and of *Aedes aegypti* larvae (Syafruddin *et al*., 1990), that were treated with pyriproxyfen.

(Federici 1993) found that the ingestion of toxicant by the insects release a toxic peptide, which binds to sites on the microvilli membranes of the midgut causing cytolysis. This latter process leads to paralysis and subsequently death of the insect. All toxicants are not equally lipophilic and many are polar compounds. In general, polar compounds are not freely permeable to cell membrane. Polar nature of toxicants may possibly denature cellular proteins. The coagulated fibrous and reticular type of cytoplasm, particularly in case of treatment with entomopathogenic fungal toxins, may account for the denaturation of the cellular proteins. Fungal toxins may have calcium-binding properties and may disorganize the cementing substance between cells and tissues, resulting in exfoliation of epithelium from underlying muscularies. Lipophilic properties of fungal toxins will obviously affect the lipid layers of the membrane, which may ultimately destroy specific permeability properties of plasma membrane. This may lead to water loss causing dehydration and possibly vacuolization. Shrinkage due to dehydration may lead to the collapse of the lumen of midgut epithelium and to the appearance of gaps among the adjacent microvilli. Exudation of cytoplasmic particles may be an attempt by the cells to eliminate toxic metabolic intermediates produced by the poisoning action of the fungal toxins. Nuclear pycnosis and disorganization of the nuclear material may be produced by the denaturation of the nuclear protein particularly shistones and DNA molecules.

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تأثير بعض المنتجات الثانوية الأيضية لثلاثة فطريات ممرضة للحشرات علي دودة ورق القطن

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2. *كلية العلوم جامعة الأزهر(بنين) القاهرة*
3. *كلية الزراعة جامعة الأزهر- القاهرة*

تعتبر المكافحة البيولوجية للحشرات إحدى طرائق المكافحة الحيوية التي استخدمت بفاعلية منذ زمن طويل وتعتبر الآن في دائرة الاهتمام في ضوء حتمية التقليل من تلوث البيئة.

تم تقدير فعالية السموم المستخلصة من العزلات الفطرية واستخدامها كمبيدات حيوية لمكافحة دودة ورق القطن الكبرى فى ظروف معملية على العمر اليرقى الأول لدودة ورق القطن الكبرى وأوضحت النتائج المتحصل عليها أن أكثر هذه الفطريات فعالية هو فطر *Metarhizium anisopliae* مسجلاً نسبة موت (84.92%) بينما كانت نسبة الموت لفطر *Paecilomyces fumosoroseus* هى (66.32%) وكانت نسبة الموت لفطر *Beauveria bassiana* هى (62.65%).

وكان الوقت اللازم لقتل 50% من الحشرات (LT_{50}) الذى أحدثته العزلة الأولى *Metarhizium anisopliae* هو (3.7211, 4.7721, 6.4808, 8.5414) بينما كان الوقت اللازم لقتل 50% من الحشرات للعزلة الثانية *Paecilomyces fumosoroseus* هو (4.7721, 64808, 7.7757, 11.0488) أما بالنسبة للعزلة الثالثة *Beauveria bassiana* كان الوقت اللازم لقتل 50% هو (5.6922, 6.8299, 8.3415, 10.4380) وكانت التركيزات المستخدمة لكل فطر (25, 50, 75, 100%).

أحدثت معاملة الطور اليرقى الرابع لدودة ورق القطن بالمنتجات الأيضية لفطر الـ *Metarhizium anisoplia* تغيرات هستولوجية شديدة فى جدار الجسم والأجسام الدهنية والمعى الأوسط ليرقات الطور السادس المتأخر من تلك المعاملة. أحدثت تلك المعاملة تلفاً فى تكوين طبقة الجليد الداخلى كما حدث تقلصاً وتحللاً للأجسام الدهنية فى المعى الأوسط ليرقات العمر السادس وقد كانت التغيرات السائدة هى تكوين فجوات فى الخلايا الطلائية مع إحداث تلف فى الغشاء المحيط بالغذاء.

أظهرت الدراسة الهستولوجية الدقيقة زيادة العضيات الليسوسومية فى الخلايا الطلائية العمودية للمعى الأوسط مع أحداث تلف فى الخملات الدقيقة وتكوين فجوات فى سيتوبلازم الخلايا العمودية.