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RESEARCH ARTICLE

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BIOCHEMICAL EFFECTS OF SOME BIOLOGICAL AGENTS ON *Culex pipiens* LARVAE (DIPTERA: CULICIDAE)

ABSTRACT

The biochemical effects of Lc25 of some biological agents, abamectin (ivermectin), *Bacillus thuringiensis* and spinosad were tested in the laboratory against the 2nd instar larvae of *Culex pipiens*. The obtained results clearly indicated that the tested biological agents decreased the total protein and content of the amino acids after 72 h. from treatment. Abamectin was more effective than *B. thuringiensis* and spinosad. It is clear from the present investigation that abamectin was considered as the promising and effective biological agents than the other two tested biological agents (*B. thurigiensis* and spinosad).

Key words : Abamectin- (Ivermectin). *Bacillus thuringiensis*-spinosad.

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INTRODUCTION

Mosquitoes are vectors of serious human diseases all over the world. Mosquito–borne pathogens infect more than 600 million people annually (Kolberg, 1994).

Many species of mosquitoes can act as vectors of *Wuchereria bancrofti*, Nile virus and Japanese encephalitis. In urban areas, the vector is commonly *culex pipiens*, but in rural areas it is often an anopheline mosquito, and occasionally a species of *Mansonia*.

Culex pipiens considered as the main vectors of human diseases such as the Rift valley fever virus (Darwish and hoogstraal, 1981), viral encephalitis and bird malaria (Kettle, 1995); elephanations (Gad *et al*., 1996), and Western Nile virus (Pelah *et al*., 2002).

Resistance of insects to insecticide has been defined as the ability to tolerate doses of these poisons which would prove lethal to the majority of individuals in a normal population of the same species. It is now accepted that resistance is an inherited characteristic and the gene structure responsible for it is already present in a few individuals of the population before the insecticide is used. Resistance to chemical insecticides is widely spread among large number of insect species (Georghiou and Mellon, 1983). In response to intensive use of organophosphorus insecticides against *Culex pipiens* mosquitoes, the resistance has increased largely thwarting control efforts (Bonning and Heming way, 1991).

Plant-derived natural products have been used for many years for human health and plant protection. An additional source of natural materials, with an excellent history of success for human and animal health products, has been metabolites of soil microrgansims. The most notable product to date is abamectin (avermectin, ivermectin) which is isolated from the fermentation of soil bacteria, *Streptomyces avermitilis* (Burg *et al*., 1979). Abamectin showed high toxicity to a number of insects (Dybas *et al*., 1989) Avermectin B1, acts on mediator of neurotransmission by gamma-amino butyric acid (GABA) leading to paralysis.

Avermectin are an environmentally safe group of compounds and are being used recently for the control of some arthropod borne diseases in man and animal (Hally *et al*., 1993).

The toxicity of abamectin to *Culex spp* was studied by many authors (Buss *et al*., 2002; Krishnamoorthy

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et al., 2002; Ramaiah *et al*., 2003; Richards *et al*., 2005).

Another such unconventional product is thuringiensin , the B-exotoxin produced by several varieties of *B. thuringiensis* . Thuringiensin inhibits DNA-dependant RNA polymerase, subsequently blocking cell mitosis. Thuringiensin acts as inhibitor for protein synthesis through interference of DNA-dependant RNA polymerase by structurally mimicking ATP and competing for binding site (Sebesta *et al*., 1981).

The toxicity of *B.* thuringiensis on *C. pipiens* larvae were studied by many investigators (Merdan *et al*., 1991; Becker *et al*., 1992; Eritia and Arancla, 1995; Mohamed and Abdel-Aal., 1995; Alziftawy, 1997; Abdel-Megeed *et al*., 1998; Thiery *et al*., 1999; Hafez, 2000; Lee *et al*., 2001; Lone *et al*., 2003; Saleh *et al*., 2003; Khater, 2003; Zayed and Bream, 2004; Sarrafzaden *et al*., 2005; Paul *et al*., 2005; Liu and Dean, 2006).

Another product is spinosad (Tracer) is the result of the fermentation process of a newly discovered species of actinomycete, *Saccharopolyspora spinosa* Numerous studies have shown that spinosad is highly effective against a wide spectrum of insect pests. (Sparks *et al*., 1995; Bret *et al*., 1997). Spinosad has a rapid speed of killing, residual control expected of synthetic insecticides, low mammalian toxicity, reduced environmental risk, and safety to beneficial insects often associated with biological insecticides. In addition, the unique mode of action of these products can enable effective pest control in circumstances of difficult insecticide resistance (Bret *et al.,* 1997).

Spinosad acts significantly faster than slow acting products like *Bacillus* bacteria*, Beauveria* fungi and other traditional biologicals agents. This is largely due to its contact mode of entry, rather than a strict reliance on ingestion as with most traditional bioagents. Because of the combination of contact and ingestion activity, the onset of insect control occurs quickly and is irreversible. Symptoms appear almost immediately and complete mortality occurs within hours. Spinosad is highly active through both ingestion and contact (Larson *et al*., 1994).

The mode of action of spinosad is different from all other known insect control products, which affect nicotinic, acetylcholine receptors at the postsynaptic cell .Spinosad causes neurological effects in insects that are consistent with the genera activation of nicotinice acetylcholine receptors, by a mechanism that is novel among known insecticide compounds. Spinosad has a high level of efficacy for lepidopteran larvae, as well as some Dipterous, Coleopterous, Thysanopteran and Hymenopteran, but has limited or no activity to other insects, and exhibits low toxicity to mammals and other wildlife (Mayes *et al*., 2003). The toxicity of spinosad to *C. pipiens* was studied by Cetin *et al*. (2005) and Romi *et al*. (2006).

Little information is available concerning the biochemical effects of abamectin, *B. thuringiensis* and spinosad against larvae of *C. pipiens.* Therefore, the research presented in this study was carried out to evaluate the effect of three biological agents (abamectim *B. thuringiensis* and spinosad) on the protein and amino acids contents.

MATERIALS AND METHODS

A: Insect culture and bioassays:

The mosquito, *C. pipiens* used in the present study was obtained from susceptible reared strain of Research Institute of Medical Entomology, Dokki Egypt. The colony was maintained under laboratory conditions of 27±2°c and 75±5% R.H. according to El-Bokl and Moawad (1996). The 2nd instar larvae were collected for bioassay tests. Different concentrations of the experimental biological agents were examined (0.001, 0.01, 0.1 and 1.0 ppm). In each test 25 larvae were put in a plastic cup with 100ml tap water and then treated with the agent. Each test was replicated four times. Control experiments were performed using water only. A mixture of ground dried bread and Brewer's yeast pellets (3:1) were added daily as food for the larvae. Dead larvae were daily removed and recorded. The Lc25 of the tested material was determined after 3 days from treatment according to Finney (1971).

The second instar larvae of *C. Pipiens* were treated with Lc25 of abamectin (0.0014 ppm); *Bacillus.thuringiensis* (0.4 ppm) and spinosad (0.0013 ppm.)

B- The compounds used:

The first compound used was commercial formulation of abamectin (Avermectin B1-MK 936 11M12) 1.8% wt/vol emulsifiable concentrate. This compound is a natural product of soil bacteria, *Streptomyces avermitilis.*

The second compound used was thuringiensin (B-exotoxin) of *B. thuringiensis* (32000 IU/mg). Active Ingredient 9.4 % and Inert Ingredient (Carrier) 90.6%. The third compound was spinosad which is metabolite of actinomycete, *Saccharopolyspora spinosa.* It is the active ingredient in tracer Naturalyte insect control, the first product within naturalyte class of insect control products to be marketed worldwide by Dow Elanco. The tested materials were diluted with water to stock solutions and appropriate water-diluted concentrations were prepared freshly before treatments.

C- Estimation of proteins and amino acids:

After 72 hours from treatment with Lc25 of the tested biological agents, some larvae were used to estimate the total protein and amino acids contents.

(i) : Estimation of protein

Whole body homogenate of the treated and control larvae were determined using assay colorimetric kits (Diamond, Egypt) according to the manufacturer's protocols. The absorbance of samples and standard were measured by using a spectronic 21 D, Milton Roy Spectrophotometer at 546nm.

(ii) Estimation of amino acids:

20 milligrams protein was hydrolysed according to the method described by Ibrahim and El-Eraqy (1996). The samples were analyzed by Lc3000 Amino Acid Analyzer (Flow rate: 0.2ml/min), pressure of buffer from 0 to 50 bar, pressure of reagent from 0-150, Reaction temperature 123°c.

D- Calculation and Data Analysis:-

(i): The reduction in percentage of amino acids was calculated according to Khazanie (1979).

(ii): the data analyses were made by T test.

RESULTS AND DISCUSSION

1- Effect of biological agents on the total protein contents:

It is quite clear from the obtained in results that there is a marked decrease in the total protein content in the whole body homogenate of *C. pipiens* larvae treated with Lc25 of the tested biological agents as compared to the control (Table 1). The total protein content was 3.6, 3.7, 4.9, when treatment carried out with abamectin, *B. thuringiensis,* and spinosad respectively, as compared to 6.1mg/gm body weight in control group. Also, these results indicated that abamectin was more effective on the protein than *B.thuringiensis* and spinosad.

Table 1. Effect of Lc25. of the tested biological agents on the total protein content of treated 2nd instar larvae of *Culex pipiens*

Biological agents	Mean body protein content ±S.E. (mg . protein / gm body weight)	
	3rd instar larvae	% Decrease
Control	6.1 ± 0.004	-
Abamectin	3.6 ± 0.01**	- 40.98
Bacillus thuringinesis	3.7 ± 0.013**	- 39.34
Spinosad	4.9 ± 0.01*	- 19.67

* Significant

* Significant

** Highly significant

Lc25 of abamectin (0.0014 ppm)

Lc25 of *B. thuringinesis* (0.4 ppm)

Lc25 of Spinosad (0.0013 ppm)

Proteins are essential constituents of the general animal cells and also in the maintenance of different activities. *B. thuringiensis* and abamectin decreased the total protein content in the midgut and the fat cells of the larvae of *Spodoptera littoralis* Abou-El-Mahasen (2007). Assar (2004a) stated that abamectin reduced the protein content in the larvae of *Musca domestica*. These changes may be due to certain defects in enzymes that are responsible for protein and lipid synthesis. On the other hand, Assar (2004b) stated that the total protein content in the larval midgut and the fat bodies of *Parasarcophage aegyptiaca* increased after 5 days from treatment with abamectin and spinosad. Abamectin and thuringiensin decreased the digestive enzymes (invertase, amylase and trehalase) activities of *Spodoptera littoralis* larvae Abou- El-Ghar *et al*. (1995). Because protein is essential to chitin synthesis, the depletion of these metabolic macromolecules indicates that chitin production must be inhibited. Also, proteins are essential for energy production; these fertility and fecundity of the adult are affected. The obtained results agree with the mode of action of these materials. *B. thuringiensis* inhibits protein synthesis through interference of DNA-dependent RNA polymerase by structurally mimicking ATP and compete for the binding site (sebesta *et al*., 1981). Abamectin acts on the mediation of neurotransmission by α-amino butyric acid (GABA) leading to paralysis.

The insect body contains thousands of different types of proteins , each with a very specific purpose. A protein may be merely structural giving form and strength to the exoskeleton or binding cells together into biochemical reaction, the storage and transport of a nutrient or waste product of the movement of a specific molecule across cell membranes. Most insecticides currently in use act on target proteins involved in nervous system signaling (neuroactive agents), cellular respiration (respiration disruptors), or growth and development (insect growth regulators). Some target proteins contain more than one target site to which insect control products bind to cause their deterimental effects. The effect of binding on the target protein (inhibition, activition, etc.) and how this effect leads to symptoms is known as the mode of action. Mode of action of an insect control product is important because it helps determine safety, speed of action and resistance (Salgado, 1997).

2- Effect of biological agents on the amino acids:

The chemical analysis of larval body using the Amino Acid Analyzer indicated that the body of *C. pipiens* larvae contained 16 different free amino acids (Table 2-4 and Figs 1-4). Data presented in table 2 and figures 1-4 shows that the time of appearance of the amino acids by Amino Acid Analyzer. Aspartic acid was separated firstly after 11min in the control, followed by threonine, seriae, glutamic, praline , glycine, alanine, cystin, methionine, isoleucine, tyrosine and phenylalanine. On the other hand, histidine, lysine and arginine were separated lastly at 50, 53.47 and 61.6 min, respectively. The amino acids in treated groups were separated at the same time of the control group.

Table 2. The appearance of different amino acids

Time (min) / Amino acids	Control	Abamectin	*Bacillus thuringinesis*	Spinosad
Aspartic acid	11.00	10.83	10.95	10.97
Threonine	14.53	14.33	14.38	14.53
Serine	15.93	15.72	15.78	15.92
Glutamic acid	17.58	17.33	17.47	17.52
Proline	20.42	20.17	20.25	20.38
Glycine	25.57	25.32	25.40	25.57
Alanine	26.72	26.45	26.27	26.72
Cystin	32.38	32.23	32.27	32.40
Methionine	37.85	37.73	37.70	37.93
Isoleucine	38.90	38.82	38.78	38.98
Leucine	41.42	41.38	41.38	41.47
Tyrosine	42.95	42.92	42.92	43.00
Phenylalanine	46.10	46.02	46.00	46.18
Histidine	50.00	49.82	49.73	50.12
Lysine	53.47	53.45	53.38	53.50
Arginine	61.10	61.45	49.42	60.57

Results presented in tables 3 and 4 showed that the most abundant amino acids in the body of the untreated and treated larvae of *C. pipiens* were, glutamic, aspartic, cystin and alanine this was followed by Lysine, serine, arginine, glycine, therionine, Isoleucine, histidine, tyrosine, methionine and leucine, however phenyalanine was considered the lowest.

It is clear from results obtained in table 4 the percent concentration of 16 tested amino acids greatly

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decreased as a result of treatment of 2nd instar larvae of *C. pipiens* with Lc25 of the tested biological agents. This decrease was more pronounced with abamectin than *Bacillus thuringiensis* and spinosad.

The percent reduction of glutamic acid content was 46.29, 13.34 and 10.66 with abamectin, *B.thuringiensis* and spinosad, respectively. The corresponding figure of aspartic acid content was (53.35, 15.02 and 12.03) with abamectin, *B. thuringiensis* and spinosad, respectively. The same trend was also determined for cystin content (60.7, 24.83, and 18.08) when treatments carried out with abamectin, *B.thuringiensis* and spinosad, respectively. In the case of alanine content, the percent reduction was 58.92, 11.56 and 7.66, respectively. Again, it is quite clear from the obtained results that the percent reduction of the other amino acids was higher with abamectin than *B. thuringiensis* and spinosad. Krap (1979) reported that there are 20 different amino acids commonly in dipeptides and polypeptide chain proteins. Proteins are composed either wholly of amino acids or of amino acids bound together with some types of molecules. There are miscellaneous functions that require specific proteins. Also, proteins provide structure support both within the cell and in the extracelluar space. Therefore the amino acids might give a clear picture about the effect and function of the used materials on protein via presence, increase or / and reduction of these amino acids.

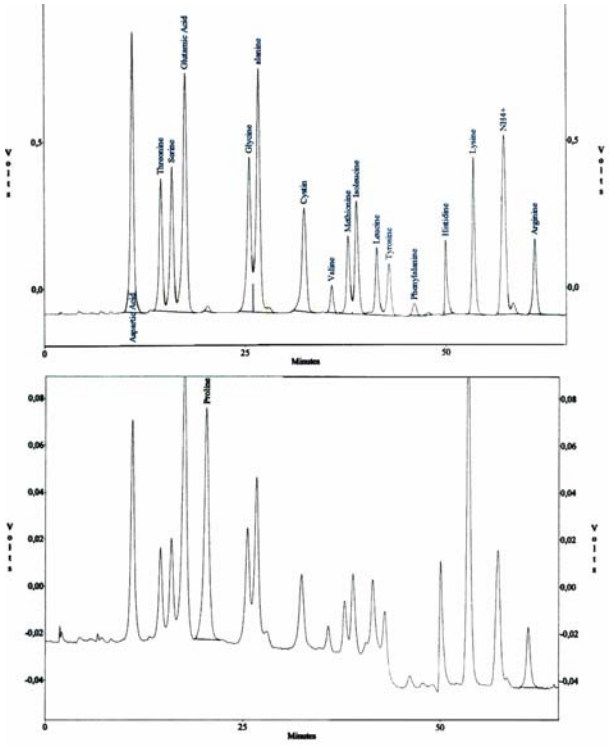

Fig 1. Amino patern in the whole tissues of untreated larvas of *Culex pipiens*

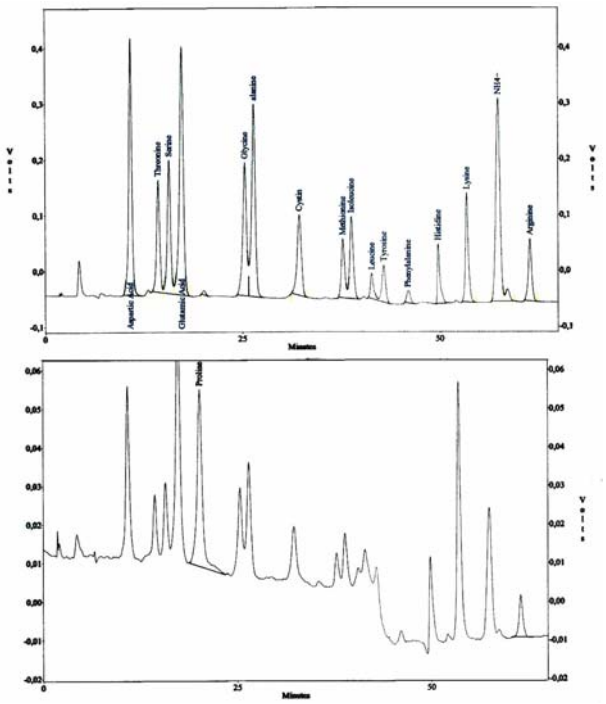

Fig. 2. Amino acids pattern in the whole body tissues of Culex pipiens larvas treated with 0.0014 PPm of abamectin.

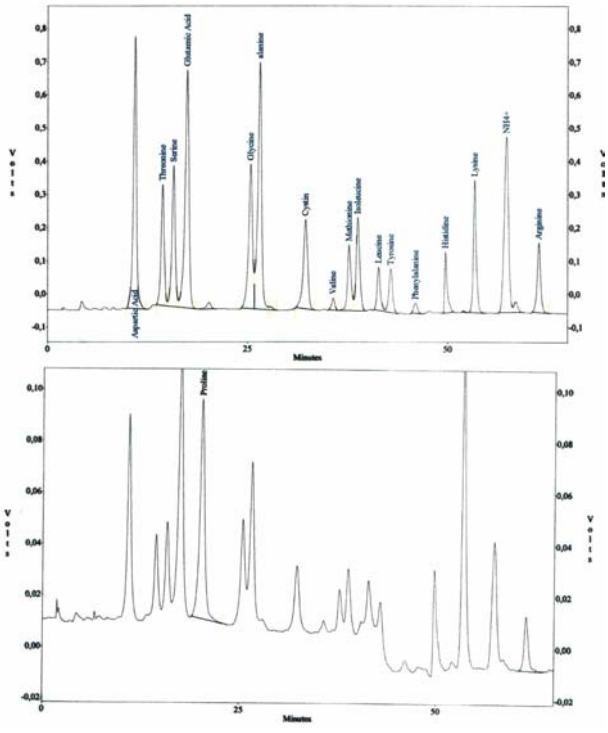

Fig. 3. Amino acids pattern in the whole body tissues of Culex *pipiens* larvas treated with 0.4PPm of *Bqcilus c.huringiensis*

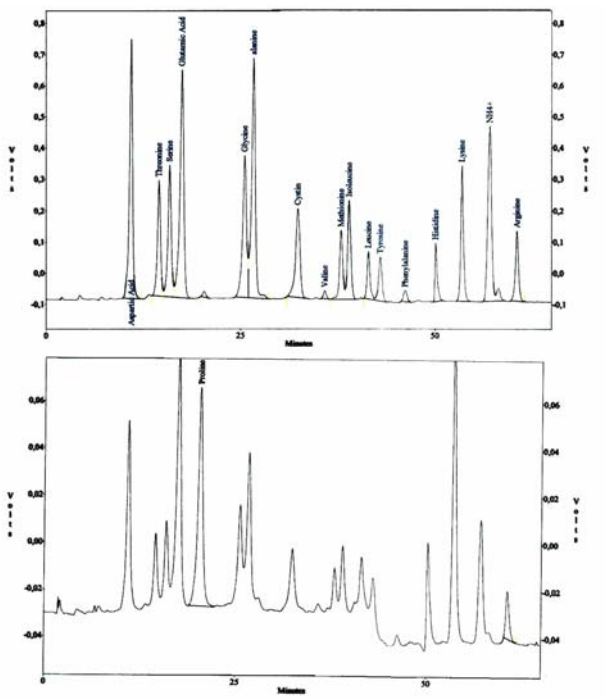

Fig. 4. Amino acids pattern in the whole body tissues of Culex pipiens larvas treated with 0.01 PPm of spinosad.

Table 3. Effect of Lc25 of the tested biological agents on the amino acids content of the larvae tested as 2nd instare

Biological agents / Amino acids		Control	Abamectin	Bacillus	Spinosad
		Conc. µg/ml.	Conc.µg /ml.	Conc. µg/ml.	Conc. µg/ml.
I.Acidic	Glutamic acid	643.01	345.34	557.23	574.43
	Aspartic acid	443.29	206.76	376.67	389.96
	Total	1086.30	552.20	933.90	964.39
II Basic	Lysine	228.46	86.98	176.02	183.15
	Arginine	183.16	80.39	155.07	153.81
	Histidine	113.35	54.19	88.98	83.78
	Total	524.97	221.56	420.07	420.74
III Neutral	Cystin	446.74	175.55	335.81	365.94
	Alanine	262.60	107.85	232.23	242.46
	Proline	224.5	112.99	193.62	213.06
	Serine	191.57	90.41	159.84	163.03
	Glycine	155.89	68.38	126.33	135.29
	Threonine	152.29	65.11	117.95	122.55
	Isoleucine	148.36	57.62	111.55	117.28
	Tyrosine	111.55	40.75	82.08	85.12
	Methionine	99.44	38.57	76.99	81.00
	Leucine	97.89	18.32	52.51	57.76
	Phenylalanine	22.63	11.35	16.95	19.96
	Total	1913.46	786.63	1505.86	1603.45
Total of all amino acids		3524.73	1560.39	2859.83	2988.58

Table 4. Effect of Lc25 of the tested biological agents on the amino acids content of *Culex pipiens* larvae

Biological agents / Amino acids		Control		Abamectin		Bacillus thuringinesis		Spinosad	
		%	% reduction	%	% reduction	%	% reduction	%	% reduction
I.Acidic		18.24	-	22.13	46.29	19.48	13.34	19.22	10.66
Glutamic acid		12.57	-	31.25	53.35	13.17	15.02	13.04	12.03
Aspartic acid									
II Basic	Lysine	6.48	-	5.57	61.92	6.15	22.95	6.12	19.83
	Arginine	5.19	-	5.15	56.10	5.42	15.33	5.14	16.02
	Histidine	3.21	-	3.47	52.19	3.11	21.52	2.80	15.33
III Neutral	Cystin	12.67	-	11.25	60.70	11.74	24.83	12.24	18.08
	Alanine	7.45	-	6.90	58.92	8.13	11.56	8.14	7.66
	Proline	6.36	-	7.24	49.67	6.77	13.75	7.13	5.09
	Serine	5.43	-	5.79	52.80	5.58	16.56	5.45	14.89
	Glycine	4.43	-	4.38	56.13	4.44	18.96	4.53	13.21
	Threonine	4.43	-	4.17	57.24	4.13	22.54	3.10	19.52
	Isoleucine	4.25	-	3.69	61.16	3.90	24.81	3.92	20.94
	Tyrosine	3.16	-	2.63	63.46	2.87	26.41	2.84	23.69
	Methionine	2.84	-	2.47	61.21	2.69	22.57	2.74	18.54
	Leucine	2.77	-	1.17	81.28	1.83	46.35	1.93	40.99
	Phenylalanine	0.64	-	0.72	49.84	0.59	25.09	0.66	11.79

Amino acids are required for the production of structural proteins and enzymes (they are present in the diet as proteins). Proteins or amino acids are always essential in the diet. Although some 20 amino acids are needed for protein production, only 10 are essential in the diet, the others can be synthesised from these ten as in other animals. Insects cannot synthesize certain amino acids and many other organic compounds they need, but obtain them by eating other living or dead organisms or green plants. The ten essential amino acids are arginine, lysine, leucine, isoleucine, tryptophan, histidine, phenylalanine, methionine, valine and threonine. In general the absence of any one of these essential acids prevents growth. Although other amino acids are not essential, they are necessary for optimal growth, glutamic and aspartic acids are necessary in addition to the essential amino acids for good growth (Chapman, 1988).

Proline is known to be a possible energy reserve since it is a derivative of glutamic acid and could enter the citric acid cycle after deamination to α-ketoglutaric acid. The varying concentration of proline at different dose levels is probably due to the varying rates of utilizing the amino acid as a source of energy in repair mechanism (Bursell, 1963).

Chen (1974) reported that alanine is a very active transaminase and plays an important role in glucose production from pyruvic acid through transamination. The glutamic acid alanine transaminase system serves as the main pathway in both the deamination of glutamic acid to ketoglutaric acid and the conversion of pyruvic acid to alanine.

Hussein *et al*. (1976) showed that arginine was the most abundant amino acid in the spingy boll worm larvae. Choe (1982) stated that cystin is not always an essential amino acid for animals, even though; methionine is still considered essential for them because of the transformation from the latter to the former.

Gohar *et al*. (1978) estimated the effects of dursban, cyolane and lannate on the amino acids content of treated larvae of *Spodoptera littoralis*. They found that the sublethal doses of these insecticides caused disturbance in the percentage of amino acids in free form. The sublethal doses of dursban and lannate tended to increase the simple amino acids (glycine, alanine, valine and leucine), while all insecticides decreased the basic amino acids (lycine, arginine and tryptophan). Salem *et al*. (1980) found 16 amino acids in the haemolymph of *Philosamia ricini* larvae. The concentration levels of amino acids were lower in larvae infected by *B. thuringiensis* and starved; where as treatment with antibiotic tetracycline resulted in higher total free amino acids. Sbalasur (1981) mentioned that *Heliothis armigera* larvae treated with sumithion induced significant decrease in free amino acids content as compared to control. Prasad and Nath (1986) stated that endosulfan and malathion caused decline in the amino acids content and the effects were greatest in the youngest larvae and lowest in the prepuae of *Spodoptera litura.*

Abou El-Ela *et al*. (1993) showed that treatment of *Synthesionyia nudiseta* larvae with dimilin baysir and altosid induced some variations in the amino acids of the resulting pupae. Zeenath and Nair (1994) concluded that when the 6th instar larvae of *Spodoptera mauritia* were treated with Juvenile hormone analogue, the total amino acid concentration increased. Tayeb *et al*. (1996) stated that aspartic, glutamic, proline and histidine were the predominant amino acids in the Haemolymph of *Agrotis ipsilon* larvae; El-sayed *et al*. (1996) studied the effect of some extracts on the larvae of *Bombyx mori* and found that garlic (0.01%) increased alanine and tryrosine; black cumin (20%) increased pheynylanine and therionine, while Rosella (10%) increased glycine, tyrosine and phenylalanine.

Khalaf (1998) studied the effect of two volatile oils of *Cymbopogon citratus* and *Rosmarimus efficinatis* on the second instar larvae of *Muscina stabulans*. These volatile oils induced various degrees of disturbance in the amino acid composition of pupae treated as larvae, in the following manner. Amino acids increased by oil treatment were (glycine, alanine valine, leucine, isoleucine, phenylalanine, tyrosine and proline). Leucine and tyrosine were the domininat oil treatment with B-Amino acids decreased (histidine, lysine, arginine, aspartic and glutamic acid) C-Amino acid were not affected. The differences in toxicological activites of *C. citratus* and *R. efficianlis* may be due to the variation in photochemical structure of these oils. Assar (2004a) stated that abamectin reduced the amino acids content in the larvae of *Musca domestica*. On the other hand, Assar (2004b) stated that the amino acids contents in the larval midgut and fat bodies of *Parasarcophaga aegyptiaca* increased after 5 days from treatment with Lc25 abamectin and spinosad. The most abundant amino acids in the body of the untreated larvae were glutamic, proline and isoleucine. These were followed by alanine, aspartic, valine and tyrosine. All the tested amino acids greatly increased by abamectin and spinosad treatment. This increase was more with spinosad than with abamectin.

CONCLUSION

It is obvious that the three tested biological agents, abamectin, *B. thuringiensis* and spinosad decreased the protein and amino acids content in *Culex pipiens* larvae. The differences in biochemical effects of these agents may be due to the variation in the chemical structures. Because both protein and glucose are essential to chitin synthesis, the depletion of these metabolic macromolecules indicates that chitin production must be inhibited. It is well known that the proteins are the major and inessential for the insect life i.e. for energy, production and for the adult fecundity and fertility which affected clearly by this organic matter. Finally, one can say that the tested agents may be used as promising agents for controlling *Culex pipiens* which considered one of the most insect vectors of human diseases. Also, the protein is essential for energy production, the fertility and the fecundity of adult will affect. Finally we can say that the tested agents may be used for controlling this vector *(Culex pipiens*) larvae.

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التأثيرات البيوكيميائية لبعض العوامل الحيوية على يرقـــات بعوضـة Culex Pipiens (ذات الجناحيــن – كيوليسيدى)

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تم دراسة التأثيرات البيوكيميائية للعوامل الحيوية abamectin (avermectin) و Bacillus thuringiensis وSpinosad في المعمل على يرقات العمر الثاني ليعوضة Culex pipiens بتركيز LC25. ولقد أشارت النتائج بوضوح أن العوامل الحيوية المختبرة أدت الى نقص المحتوى البروتيني والأحماض الأمينية بعد 72 ساعة من المعاملة . ولقد لوحظ أن (avamictin) كانت أكثر فاعلية إذا قورن بـ(Bacillus thuringiensis) و (spinosad)

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