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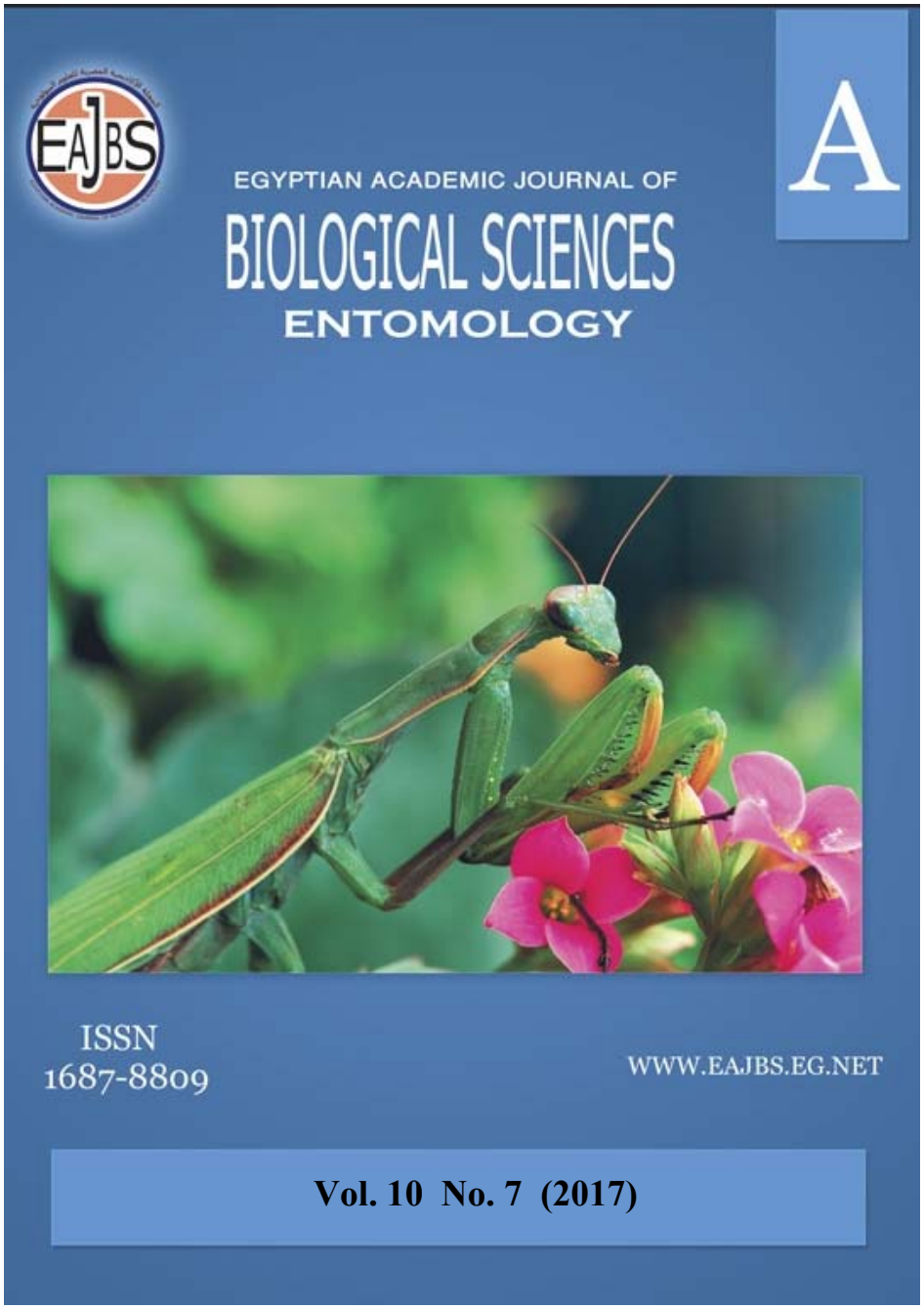

Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences, Department of Entomology, Faculty of Sciences Ain Shams University.

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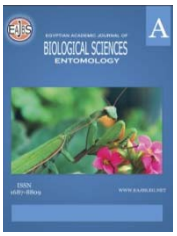
www.eajbs.eg.net

Egypt. Acad. J. Biolog. Sci., 10(7): 311–322 (2017)

Egyptian Academic Journal of Biological Sciences
A. Entomology

ISSN 1687- 8809
www.eajbs.eg.net



Insecticides Application and the Egyptian Cotton Leafworm, *Spodoptera littoralis* (Boisd.) Permanent Larvae

Hassan F. Dahi[1] ; Abdel-Rahman G. Abdel-Rahman[2]; Mahmoud M. El-Bamby[3]; Walaa E. Gamil[1] and Doaa S. Rasheed[2]

1- Plant Protection Research Institute, Agricultural Research Center, Doki, Giza, Egypt
2- Plant Protection Department, Desert Research Center, Mataria, Cairo, Egypt
3- Environment and Bio-Agriculture Department, Faculty of Agriculture, Al Azhar University, Cairo, Egypt

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ARTICLE INFO

Article History
Received: 15/10/2017
Accepted: 15/11/2017

Keywords:
Permanent larva, *Spodoptera littoralis*, insect growth regulators (IGR), Spinetoram, Molecular biology, Biochemical effects, Amino acids, Genotype

ABSTRACT

Permanent larva phenomenon is the elongation in the larval stage. This phenomenon occurs as a result of imbalance in insect hormones after treating the larvae with some insecticides such as insect growth regulators (IGR). This work aimed to explain this phenomenon as a result of treating *S. littoralis* 4th instar larvae with Spinetoram, Lambda-cyhalothrin, Chlorpyrifos and Emamectin Benzoate. Highly significant increase in larval duration was recorded in treated larva. Significant changes in total carbohydrates, total protein and total lipid contents as well as the main enzymes activities were recorded in the permanent larvae. Genetic differences between normal untreated *S. littoralis* and permanent larvae were also studied.

INTRODUCTION

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) is one of the most notorious and destructive phytophagous insect pests in Egypt, not only to cotton, but also to other field crops and vegetables (Kandil *et al.,* 2003). These caterpillars are very polyphagous, causing important economic losses in both greenhouses and open field on a broad range of ornamental, industrial and vegetable crops. Besides many populations have acquired resistance towards most insecticide groups (Alford 2000).

Treatment of insects with insecticides may result in decrease in egg production, affect egg fertility, elongate larval or pupal duration, suppression of weight, insect malformations, or reduction in reproductive capacity(Hewady 1990, , Knight 2000, Radwan 2001 and Pineda *et al*. 2004). Many researchers reported the elongation of larval duration but finely it developed to larva-pupa intermediate stage or developed to normal pupa (Abdel-Rahman *et al.* 2003, El-Barkey *et al.* 2009 and Gamil 2012). Permanent larva phenomenon is the elongation in the larval stage and the larva does not develop to pupae. This phenomenon occurs as a result of hormonal imbalance in insect when the larvae treated with some insecticides such as insect growth regulators (IGR). Wigglesworth (1972) said that the progressive development of immature (larval and pupal characters) and mature stages is

controlled by the hormonal balance between both Ecdyson and Juvenile hormones. Accordingly treating larva with an Ecdyson analogue may interfere with such balanced hormonal system causing morphogenetic defects in the developmental sequence. Vogel *et al.* (1979) analyzed the reactions of immature insects of more than 40 species placed in continuous contact with high doses of juvenile hormone active insect growth regulators. 4 different types were recognized: Inhibition of both ecdysis and metamorphosis, defective metamorphosis, defective adult emergence and defective embryogenesis. Abdel-Rahman *et al.* (2002) mentioned that increasing the applied Neem Azal doses led to an elongation in the treated *Pectinophora gossypiella* larval period in an ascending order and finally ended by the formation of permanent larvae at the higher administered doses. In previous studies on development of resistance of cotton leafworm against certain insecticides, treating larvae with Spinetoram for six generations caused an elongation in larval period and the last larval instar could not develop to pupae and died as permanent larvae. When the larvae were treated with Emamectin or Chlorpyrifos , they could not develop to pupae and the phenomenon of permanent larvae was appeared from the first generation, while in case of Lambda-cyhalothrin it was recorded in the second generation (Rasheed 2017, Rasheed *et al*. 2015 and Rasheed *et al*. 2016) . This work aimed to explain this phenomenon as a result of treating the cotton leafworm, *S. littoralis* larvae with Spinetoram pesticide.

MATERIALS AND METHODS

Insects rearing technique:

The basis of t he culture designed to provide cotton leafworm, *S. littoralis* used in the present work was obtained from freshly collected eggs masses supplied from the Cotton Leafworm Department, Plant Protection Research Institute, Dokki, Egypt. Larval stages were reared on caster bean leaves (*Ricinus communis*) which were provided daily. The formed pupae were collected and placed in clean jars with moist saw dust placed at the base to provide the pupation site. Adults were provided with 10% sugar solution. All stages of *S. littoralis* were cultured and tested at 27±2°C and 70± 5 % R.H.

Buildup of resistant strains Spinetoram insecticide:

Spinetoram is a mixture of major and minor components:

Major component (3'-O-ethoxy-5,6-dihyro spinosyn J)

Minor component (3'-O-ethoxy spinosyn L)

- Common name: Spinetoram
- Trade name: Radiant.
- Formulation used: (12% SC.)

The leaf-dipping bioassay method was used to determine the median lethal concentration (LC_{50}) values. Serial concentrations of the pesticide were prepared. For each concentration, caster bean leaves were dipped for 30 seconds then allowed to dry at room temperature. Treated leaves were introduced to 4^{th}instar larvae of the cotton leafworm for 24 h after that mortality counts were recorded. Mortality percentages were corrected using Abbott's formula (Abbott 1925). The LC_{50} values were calculated according to the method of Finney (1971). Insects were maintained under constant conditions of 25 ± 2 °C and 65 ± 5% R. H. and any surviving larvae were transferred to clean jars containing fresh untreated leaves to have the following generation. Using the LC50 value obtained from the previous generation, the same technique of selection and rearing was followed at each successive generation. For

each tested generation, larval duration (from the initial treated instar up to pupation) was calculated.

Biochemical studies:-

Whenever the tested treatments produced permanent larvae the following biochemical determinations were done.

Total carbohydrates were extracted and prepared for assay according to Crompton and Birt (1967) and Total carbohydrates content were estimated in acid extract of sample by the phenol-sulphuric acid reaction of Dubios *et al.* (1956).

For other biochemical measurements; the surviving larvae exhibiting toxic symptoms were anaesthetized and rinsed with 5 ml acetone to remove surface residues, the larvae were weighed then homogenized in phosphate buffer (pH 7) using a Teflon tissue homogenizer surrounded by crushed ice. The homogenates were centrifuged at 8000 rpm for 20 min at 4^0 C and the supernatant was used directly for the determination of the following:

∗ Total protein content of the total body was determined according to Bradford (1976).

∗ Total lipids were estimated by the method of Knight *et al*. (1972).

∗ Glutathione-S-transferase activity was determined according to the method of Habig *et al.* (1974).

∗ Non-specific α and β esterase activity was measured as described by Van Asperen (1962).

∗ Acetyl choline-esterase (AChE) activity was determined using acetylcholine bromide (Ach Br) as substrate according to the method described by Simpson *et al.*(1964).

∗ Acid and alkaline phosphatase activity was measured from the larval hemolymph as described by Powell and Smith (1954).

∗ Mixed Function Oxidase (MFO) activity was determined according to the method of Hansen and Hodgson (1971).

∗ Total Amino Acids (HCl- hydrolyzed) were determined according to Pellet and Young (1980)

Molecular biology assay:

Preparation of PCR Reaction:

Random amplified polymorphic DNA (RAPD) is a PCR based technique for identifying genetic variation. It involves the use of a single arbitrary primer in a PCR reaction, resulting in the amplification of many discrete DNA products. Preparation of buffers, isolation and purification of genomic DNA and preparation of PCR reaction were done depending on Sambrook *et al.* (1989), Williams *et al.* (1990), Walsh *et al.*(1991), Rohland and Hofreiter (2007), Lagisz *et al.* (2010) and Swain *et al.* (2010). Subset of 5 random primers were used in the detection of polymorphism among the samples. The nucleotide sequence of five primers used for RAPD-PCR analysis was shown in table (1).

Statistical analysis:

The significance of the main effects was determined by one way analysis of variance (PROC. GLM). The significance of various treatments was evaluated by Tukey's multiple range tests ($p< 0.01$). All analyses were made using the Statistical Analysis System Version 9.1 program PROC PROBIT **(SAS Institute 2003).**

Table (1): Nucleotide sequence of the five primers used for RAPD-PCR analysis

No.	Primer	Primer sequence
1	OPA-03	AGTCAGCCAC
2	OPA-04	AATCGGGCTG
3	OPA-05	AGGGGTCTTG
4	OPA-06	GGTCCCTGAC
5	OPA-08	GTGACGTAGG

RESULTS AND DISCUSSION

Larval duration of permanent larvae:

Data in **Table (2)** appeared that the *S. littoralis* larval duration (from 4th larval instar till death) of permanent larva was 23.67 days compared with 10.93 days of untreated larvae (from 4th larval instar till pupation) showing a highly significant increase in larval duration by 116.5%.

Table (2): Duration of *S. littoralis* larvae (from 4th instar until pupation or death of the larvae).

Strains	Larval duration (days)	% Increase than control**
Untreated larvae	10.93±0.23	—
Permanent larvae	23.67±2.91*	116.5

* means highly significant (p of $t \leq 0.001$)

** Increase than control (%) = 100 (Treated - Control) / Control

This result was in agreement with many researchers who reported also the formation of permanent larvae at higher doses of AzA as Schulz and Schluter (1983) on *Epilachna varivestis* and Schluter *et al.* (1985) on *Manduca Sexta* and Imam (2001) and Abdel-Rahman *et al.* (2002) on *P. gossypiella*. The active ingredient azadirachtin is structurally similar to the ecdysones insect hormones which control the process of metamorphosis as insects pass from larva to pupa to adult (Ascher 1993). Watson and Kelly (1995) studied the amount of toxin of *Bacillus thuringiensis* required to prevent development of resistance in *P. gossypiella* and found that there was positive correlation between the used concentration and retardation in larval development, the highest used concentration (0.375 ug /ml diet) prevented all the exposed 1st instar larvae from ecdysis to the next instar even after 9 days while the control larvae developed to the 4th instar after the same period (9 days).

Growth and development of insects are under the control of hormones, including prothoracicotrophic hormones (PTTH) (brain hormone), ecdysteriods (ecdysone) (molting hormone) and juvenile hormone (JH). The peptide hormone PTTH secreted from the brain controls the secretion of the molting hormone (ecdysone) from the prothoracic gland. Ecdysone is responsible for cellular programming and together with JH initiating for molting process. JHs control a number of processes such as embryogenesis, molting and metamorphosis, reproduction, diapause, communication, migration / dispersal, caste differentiation, pigmentation, silk production and phase transformation. When JH levels secreted

from the corpora allata are low, the epidermis is programmed for a larval molt, otherwise, the epidermis is programmed for metamorphosis. JH is virtually absent in the pupae, but is present in adults to serve some functions in reproduction. Thus, JH suppresses pupation and induces vitellogenesis during the reproductive stage of the insects. Any disturbance in the normal hormone may cause a crucial disorder in growth and development of insects. (Eto 1990).

The elongation in larval periods ended by the formation of permanent larvae at higher applied doses means that, the critical concentration of the Ecdyson was reached after a considerable longer period of time compared with untreated ones. Insects poisoned with Insect Growth Regulators (IGR) or other insecticide interferes with the production of ecdysone cannot molt or reproduce and eventually they die (Brown 2005 and Childs 2005).

These results indicated that Radiant (the tested Spinetoram formulation) might cause either inhibition of Ecdyson release or interference with some transmitters involved in the regulation of Ecdyson biosynthesis and /or release causing the imbalance between secretion of Ecdyson and Juvenile hormone which led to appearance of this phenomenon.

Biochemical Aspects of Permanent larvae:

Effect on main contents:

Data in Table (3) showed non-significant different in the total carbohydrates between untreated larvae and permanent larvae of *S. littoralis*. On the other hand, significant increase in total protein content in the permanent larvae by 28.17% than untreated larvae. This increase in total protein may be due to the elongation in larval duration. The insect body contains 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 (Assar *et al.*, 2012).

Lipids are essential structural component of cell membrane and cuticle.They provided a rich source of metabolic energy. Significant decrease in total lipids content in permanent larvae by 63.92% than untreated larvae was obtained as shown in Table (3).This result is in agree with Rawi *et al.* (2011) who found highly significant decrease in the level of total lipids on the 4th Instar larvae of *S. littoralis* post treatment of with LC_{10} of *Azadirachta indica* or *Citrullus colocynthis*. The great reduction in total lipids might be due to breaking down lipids to simpler moieties that could be utilized as a carbon source for growth.

Table (3): Total carbohydrates, protein and lipid contents in *S. littoralis* untreated and permanent larvae.

Strains	Carbohydrates (mg/ml)	Increase (%)*	Protein (mg/ml)	Increase (%)*	Lipid (mg/ml)	Increase (%)*
Untreated larvae	7.37±0.27^{ns**}	–	10.40±0.26	–	8.40±0.21**	–
Permanent larvae	7.73±0.27^{ns}	0.05	13.33±0.12**	28.17	3.03±0.15	-63.92

*Increase than control (%) = 100 (Treated - Control) / Control

** means highly significant and ns means not significant (p of t ≤ 0.001)

Table (4): Acetyl Choline- esterase (AChE), Glutathione S-transferase (GSTs) and mixed function oxidase (MFO) activities in untreated and permanent larvae of *S. littoralis*.

Strains	AChE *(µg AChBr /min/g. body weight)*	Increase (%)*	GSTs *(m mole sub. conjugated/min/g. body weight)*	Increase (%)*	MFO *(mmole sub oxidized/min/g. body weight)*	Increase (%)*
Untreated larvae	65.87±2.29**	—	23.67±1.86	—	31.10 ± 1.17^{ns}	—
Permanent larvae	31.33±2.06	-52.5	168.00±4.16**	609.75	35.03 ± 1.02^{ns}	12.6

*Increase than control (%) = 100 (Treated - Control) / Control
** means highly significant and ns means not significant (p of $t \leq 0.001$)

Table (5): Non- specific esterase activities in untreated larvae and permanent larvae of *S. littoralis (µg α-naphthol/min/g. body weight)*.

Strains	α-esterases	Increase (%)*	β-esterases	Increase (%)*
Untreated larvae	33.67±2.85	—	92.00±5.29	—
Permanent larvae	335.00±14.57 **	894.9	212.00±8.33**	130.43

*Increase than control (%) = 100 (Treated - Control) / Control
** means highly significant (p of $t \leq 0.001$)

Table (6): Acid and alkaline phosphatase activities (µM p-nitrophenol /g. body weight) in untreated larvae and permanent larvae of *S. littoralis*.

Strains	Alkaline phosphatase	Increase (%)*	Acid phosphatase	Increase (%)*
Untreated larvae	51.00±2.65	—	566.00±8.19**	—
Permanent larvae	161.00 ±5.57**	215.7	285.67±7.22	-49.5

*Increase than control (%) = 100 (Treated - Control) / Control
** means highly significant (p of $t \leq 0.001$)

Effect on enzymes:

Data in Table (4) showed significant decrease in acetylcholine esterase (AChE) activity by 52.5 % in permanent larvae than untreated larvae. A highly significant increase in Glutathione S-transferases (GSTs) activity by 609.75% was found in permanent larvae comparing with the untreated larvae. Results of the estimated Mixed Function Oxidase (MFO) activity also showed no significant difference between the two types of larvae.

Concerning the non–specific esterase activities (α-esterases and β-esterases), Table (5) showed highly significant increase in α-esterases and β-esterases activities in permanent larvae than those in untreated larvae by 894.9% and 130.43%, respectively. On the other hand, Table (6) presented acid and alkaline phosphatase activities in the permanent larvae and untreated larvae of *S. littoralis*. The results indicated highly significant increase in alkaline phosphatase by 215.7% and significant decrease by 49.5% in acid phosphatase activity in permanent larvae compared with untreated larvae.

Effect on amino acids content:

Amino acid analysis of larval body using the Amino Acid Analyzer indicated that the body of *S. littoralis* larvae contained 16 different free amino acids. They were the same in the permanent and the normal untreated larvae but significantly in higher quantities in the permanent larvae. As shown in Table (7) the increases in amino acid contents in the permanent larvae than the normal untreated larvae were arranged between 275.44% and 203.28% in Histidine and Lysine amino acids and 8.15% and 65.15% in Argenine and Tyrosine amino acids, respectively. These results were in agreements with the previous results which showed significant increase in total protein content in the permanent larvae by 28.17% than untreated larvae. This increase in total protein may be due to the elongation in larval duration.

Zeenath and Nair (1994) reported that when the sixth instar larvae of *Spodoptera mauritia* were treated with juvenile hormone analogue hydroprene, the total amino acid concentration increased. Bakr *et al.* (1991) mentioned that the total pool of free amino acids in the larvae and pupae of *Musca domestica* was increased by treatment with dimilin, as an insect growth regulator. Some amino acids can utilize as a source of energy. Proline is known to be a possible energy reserve since it is a derivative of glutamic acid and could enter the citric cycle after deamination to α- ketoglutaric acid (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 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.

Table (7): Total Amino acids content in untreated larvae of *S. littoralis* and permanent larvae.

No.	Amino acid	Untreated larvae *(mg/g)*	Permanent larvae *(mg/g)*	Increase or decrease (%) than control*
1	Aspartic	10.438	27.931	167.59
2	Threonine	4.326	11.294	161.07
3	Serine	5.062	11.807	133.25
4	Glutamic	11.975	26.057	117.59
5	Glycine	3.921	8.433	115.07
6	Alanine	6.856	13.623	98.70
7	Valine	5.269	11.016	109.07
8	Methionine	1.183	2.921	146.91
9	Isoleucine	4.168	10.635	155.16
10	Leucine	6.153	13.756	123.56
11	Tyrosine	4.066	6.715	65.15
12	Phenyl alanine	3.586	8.185	128.25
13	Histidine	5.469	20.533	275.44
14	Lysine	4.908	14.885	203.28
15	Ammonia	4.073	8.962	120.03
16	Argenine	4.541	4.911	8.15
17	Proline	7.982	16.337	104.67

Molecular Aspects of Permanent larvae:

Four primers (OP-A3, OP-A5, OP-A6 and OP-A8) out of the five tested primers gave a clear difference among the *S. littoralis* untreated and permanent larvae on the basis of the amplified product pattern. Number and molecular weights of different amplified products obtained by the tested primers with *S. littoralis* untreated and permanent larvae are presented in Table (8 A, B, C abd D).

Table (8): Number and molecular size of different amplified products produced by the four tested primers with *S. littoralis* untreated and permanent larvae.

A - OPA-03				B - OPA-05			
OPA-03 5′-AGTCAGCCAC-3′				OPA-05 5′-AGGGGTCTTG-3′			
Band	**Untreated**	**Permanent**	**Ms (bp)**	**Band**	**Untreated**	**Permanent**	**Ms (bp)**
1	0	1	850	**1**	1	0	600
2	1	0	580	**2**	1	0	550
3	1	1	500	**3**	1	0	490
4	1	1	390	**4**	1	1	390
5	0	1	190	**Total**	4	1	-
Total	3	4	-				
C - OPA-06				**D - OPA-08**			
OPA-06 5′-GGTCCCTGAC-3′				OPA-08 5′-GTGACGTAGG-3′			
Band	**Untreated**	**Permanent**	**Ms (bp)**	**Band**	**Untreated**	**Permanent**	**Ms (bp)**
1	1	1	700	**1**	1	1	600
2	1	1	600	**2**	1	1	500
3	1	1	500	**3**	1	1	350
4	1	1	400	**4**	1	0	300
5	1	1	300	**Total**	4	3	-
6	1	1	200				
Total	6	6	-				

The data in Table (8 A.) showed that, the number of produced fragments by primer OPA-03 are 5 bands, distributed as 3 and 4 fragments in normal untreated and permanent *S. littoralis* larvae, respectively. The smallest size of the amplified products was 190 bp and the largest size was 850 bp. Both of them were formed in permanent larvae but not formed with the normal untreated larvae. Bands number 3 and 4 with 400 and 500 bp were formed in the two types of larvae, while the second band was formed in the normal untreated larva and not noticed in the permanent one.

Also, data in Table (8 B.) showed that, the number of produced fragments by primer OPA-05 are 4 bands, distributed as 4 and 1 fragments in normal untreated and permanent larvae of *S. littoralis*, respectively. The smallest size of the amplified products was 390 bp formed in normal untreated and permanent larvae. The largest size was 600 bp in untreated larvae.

On the other hand, data in Table (8 C) showed that, the number of fragments produced by primer OPA-06 is 6 bands, distributed as 6 fragments in both of the normal untreated and permanent larvae. The smallest size of the amplified products was 200 bp and the largest size was 700 bp in both normal untreated and permanent larvae.

Finally, Data in Table (8 D) showed that, the number of produced fragments by primer OPA-08 are 4 bands, distributed as 3 and 3 fragments in normal untreated and permanent larvae of *S. littoralis*, respectively. The smallest size of the amplified products was 300 bp in normal untreated and not found in permanent larvae fragments, while the largest size was 600 bp formed in both of normal untreated and permanent larvae.

Table (9): List of RAPD- PCR primers and the number of amplified DNA bands generated by four DNA- RAPD primers used for the identification of *S. littoralis* untreated and permanent larvae.

Primer code No.	Total No. of bands	No. of Monomorphic bands	No. of polymorphic bands	Polymorphism (%)
OPA-03	5	2	3	60
OPA-05	4	1	3	75
OPA-06	6	6	0	0
OPA-08	4	3	1	25

Data in Table (9) showed the list of RAPD- PCR primers, their sequences and the number of amplified DNA bands generated by four DNA- RAPD primers used for the identification of *S. littoral* normal untreated and permanent larvae. Monomorphism is a band of DNA that present in all individuals but polymorphism is a marker or band that present in an individual or some individuals but is absent in another individual. For primer OPA-03, total number of bands was 5 bands. Number of monomorphic bands was 2 and number of polymorphic bands was 3 bands therefore polymorphism percentage was 60%. Primer OPA-05 formed 4 bands. One of them was a monomorphic band and the other 3 bands were polymorphic bands therefore polymorphism percentage was 75%. By the same way, six bands were formed by primer OPA-06. All of these 6 bands were monomorphic bands with 0 polymorphic bands therefore its polymorphism percentage was 0%. In primer OPA-08, total number of bands was 4 bands. Number of monomorphic bands was 3 while, number of polymorphic bands was 1 bands therefore polymorphism percentage was 25%.

These results indicated that some pesticide formulations may have a hormonal actions and have the ability to interfere the hormonal balance in the insect causing either delaying or inhibition in Ecdyson biosynthesis and /or release causing the imbalance between secretion of Ecdyson and Juvenile hormone which cause prolonging larval duration, appearance of permanent larvae or larval-pupal intermediate or causing adult deformations. Permanent larvae have few lipid contents and high contents in total protein and free amino acids. DNA- RAPD - PCR primers (OPA-03, OPA-05 and OPA-08) can be used for determining the genetic differences between *S. littoral* normal untreated and permanent larvae.

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ARABIC SUMMARY

تطبيق المبيدات الحشرية واليرقات المستدامة لدودة ورق القطن

حسن فرج ضاحى[١]– عبد الرحمن جمال الدين عبد الرحمن [٢] – محمود محمد البمبى[٣]– ولاء جميل ابراهيم[١] - دعاء رشيد سلام[٢]

١- معهد بحوث وقاية النبات – مركز البحوث الزراعية – الدقى – الجيزة – مص

٢- قسم وقاية النبات - مركز بحوث الصحراء – المطريه – القاهرة – مصر

٣- قسم البيئة والزراعه الحيوية – كلية الزراعة – جامعة الأزهر – القاهرة – مصر

ظاهرة اليرقة المستدامة عبارة عن استطالة في طول العمر اليرقى. تحدث هذه الظاهرة نتيجة لخلل فى توازن الهرمونات الحشرية بعد معاملة اليرقات ببعض المبيدات الحشرية مثل منظمات النمو الحشرية. يهدف هذا العمل الى تفسير هذة الظاهرة الناتجه عن معاملة يرقات دودة ورق القطن بميدات الإسبينيتورام، اللمبادا ثيهالوثرين، الكلوروبيريفوس و الإيمامكتين بنزوات. وقد سجلت زيادة معنوية فى طول العمر اليرقى لليرقات المعاملة. تم تسجيل تغيرات معنوية فى محتوى كل من الكربوهيدرات الكلية، البروتينات الكلية و الدهون الكلية وكذلك فى نشاطات الإنزيمات الرئيسة فى اليرقات المستدامة. ايضاً تم دراسة الإختلافات الوراثية بين اليرقات غير المعاملة واليرقات المستدامه لدودة ورق القطن.