EAJBS

EGYPTIAN ACADEMIC JOURNAL OF

BIOLOGICAL SCIENCES

PHYSIOLOGY & MOLECULARBIOLOGY

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ISSN
2090-0767

WWW.EAJBS.EG.NET

Vol. 6   No. 2 (2014)

Biological and molecular aspects of the insect growth regulator lufenuron on *Spodoptera littoralis* (Bosid) (Lepidoptera:Noctuidae)

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ARTICLE INFO

ABSTRACT

The cotton leafworm, *S. littoralis*, is a major pest in Egypt causes severe quantitative and qualitative losses of cotton and other economic crops. This study examined the effect of chitin synthesis inhibitors, lufenuron (match 5%) on the 2nd instar larvae. The results showed that lufenuron caused not only mortality in larval stages, but also caused effect on pupal and adult duration, adult fecundity and egg fertility. Also lufenuron treatments have resulted in emergence of deformed pupae and adults. PCR-RFLP technique was used in this study to investigate the effect of lufenuron on the vira-like chitinase gene, one of the genes involved in the formation of chitin and concerned with development of insect. The endonucleases *Spel, EcoRI* and *Bbsl* were used to detect the molecular changes at different time intervals 6, 12, 24 and 48hrs post treatment with sublethal doses LC_{25}, LC_{50} and $LC_{90.}$The results showed significant changes in the sequences of the treated groups which were dose and time dependants. Moreover the number of RNA secondary structure stems was varied in the different studied groups and most of them differed in their nucleotide sequences, positions and free energy.

INTRODUCTION

In Egypt many crops and various vegetables are attacked by numerous insect pests. Among these, the lepidopterous insects in general and the cotton leafworm *Spodoptera littoralis* (Boisd) in particular are the most damaging. It is extremely polyphagous and always inflicts excessive damage when it occurs in masses during certain years, commonly referred to as cotton worm monsoons (Amin and Salam 2003).

The intensive use of chemical control measures to manage this pest in particular on cotton has resulted in the development of resistance to almost all classes of insecticides used (Abo-Elghar et al., 2005). There is growing interest in the use of bioinsecticides such as insect growth regulators (Atwa et al., 2010).

Chitin synthesis inhibitors (CSIs) interfere with formation of chitin and control immature stages of many pests with relatively low harm to beneficial arthropods (Consoli et al., 2001; Wakgari and Giliomee 2003). These compounds are effective suppress of development for the entire life cycle on insects (Gelbic et al., 2011). Lufenuron (match 5%), is assorted in a group among chitin biosynthesis inhibitors or IGR in general. Lufenuron exhibited an inhibitory effect on the adult emergence of cotton leafworm and had a latent effect on egg hatchability (Adel 2012).

Chitin is one of the most important biopolymers in nature. It is mainly produced by fungi, arthropods and nematodes. In insects, it functions as scaffold material, supporting the cuticles of the epidermis and trachea as well as the peritrophic membrane lining the gut epithelium. Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin-containing structures. For this purpose, insects repeatedly produce chitin synthases and chitinolytic enzymes in different tissues. Coordination of chitin synthesis and its degradation requires strict control of the participating enzymes during development (Merzendorfer and Zimoch 2003). Chitinases (β -1,4-poly-N-acetylglucosaminidase; EC 3.2.1.14) are members of the O-glycoside hydrolase superfamily and are found in many species including microbes, plants, insects and mammals (Kramer et al., 1993). All insect chitinases belong to family 18 glycosylhydrolases have been detected in molting fluid and gut tissues and are predicted to mediate the digestion of chitin present in the exoskeleton and peritrophic membrane in the gut to chitooligosaccharides (Kramer and Koga 1986 and Kramer and Muthukrishnan 2005). Genes and cDNAs encoding insect chitinases have been identified and characterized from several insects including lepidopteran species such as Manduca sexta (Kramer et al., 1993), Bombyx mori and Hyphantria cunea (Kim et al., 1998) Spodoptera litura (Shinoda et al., 2001), Choristoneura fumiferana (Zheng et al., 2002) and Spodoptera frugiperda (Bolognesi et al., 2005) Spodoptera littoralis (Tyne and Possee 2005).

The aim of this work was to assess the effects of the chitin synthesis inhibitor Benzoylphenylurea derivatives, lufenuron (match5%) on some biological aspects of S. littoralis. Also to investigate the effect of the sub lethal doses (LC_{25}, LC_{50} and LC_{90}) on the vira-like chitinase gene and predict the secondary structure of RNA which resulted from the changes in the amino acids as a result of treatments.

MATERIALS AND METHODS
Insect maintenance

The colony of S. littoralis was supplied from the division of cotton leaf worm, of Plant Protection Research Institute, Dokki, Egypt. Larval stages were reared on castor bean. The culture and experiments were conducted in $27\pm2^{o}C$ and $65\pm5\%$ R.H.

Toxicological and biological studies:

A leaf dipping bioassay method was adapted to evaluate insecticidal activity of lufenuron against the 2nd instar larvae of S. littoralis. Five concentrations of lufenuron were used. Eighty of starved larvae, distributed in four replicates (20larvae/replicate) were used for each concentration, and treated according to method described by Baker et al., (2012). 24hrs inspection was carried out for all treatments and control to record mortality percentages. Then daily inspections were

carried out until adult emergence occurred. Larval mortality%, larval duration, pupation%, pupal duration and pupal malformation were recorded.

Adult emergence %, total inhibition of adult emergence %, fertility %, fecundity, sterility % and addition malformations were recorded. Adult fecundity was determined by placing one female and one male together in a glass jar of 75 c.c capacity provided with a piece of cotton soaked in 10% sugar solution and was internally covered with soft sheet of paper for oviposition. The jars were inspected daily for counting the number of laid eggs. To determine the fertility, two or three patches having not less than 100 eggs were collected during the first 3 days of oviposition and incubated under the laboratory conditions until hatching and the percentage of hatchability was recorded.

Statistical analysis:

Mortality data after 24hrs of treatment was corrected by Abbott's formula and analyzed by Probit analysis (Finney, 1971) to obtain LC_{25}, LC_{50} and LC_{90}. By using (Origen lab program version7.5) the data were expressed as means ± standard errors. The statistical significance of differences between individuals means were determined by using one way ANOVA test. Levels of significance of each experiment was stated to be significant at ($P \leq 0.05$), high significant at ($P \leq 0.01$) and very high significant at ($P \leq 0.001$). The oviposition deterrent index (O.D.I) was calculated according to Lundergren (1975).

Molecular Studies:

Isolation of DNA:

Total nucleic acid (nuclear DNA and mtDNA) was extracted from 2nd instar larvae treated with LC_{25}, LC_{50} and LC_{90} of lufenuron at different time intervals post treatment (6, 12, 24 and 48hrs). Whole larvae were homogenized in 0.5 ml STM (0.32M sucrose, 50 mMTris pH 7.25, 10 mM MgCl2, 0.5 %

NP detergent) and 0.1 ml of 0.5 M EDTA. The suspension was centrifuged at low speed for 4 min and the supernatant was removed. The pellet was resuspended in 0.5 ml STE (75mM NaCl, 25 mM EDTA, 10 mMTris pH 7.8) and 1% SDS. The homogenate was digested with 500 µg proteinase K for 1h and extracted with phenol and chloroform. The DNA was ethanol precipitated, resuspended in 60 µl 10 mMTris pH 8.0 and stored at -20ºC (Levy et al., 2002).

PCR-primers:

Nucleic acid sequence for *S. littoralis* vira-like chitinase gene was obtained from Gen Bank of the National Center for Biotechnology Information (Tyne & Possee 2005). The genetic computer programs Bioface, DNA counter and DNA Baser were used to detect the primers used, GTAAAGGAGATTGAAGGCAGTTTC 5-prime candidate and TAAACTTTTTTTCATATTAACTTACA 3-prime candidate.

PCR-RFLP analysis:

For PCR amplification, the PCR mixture for each reaction contain a total volume 50 µl of (6 µl of 250ng/µl of the total DNA extracted from *S. littoralis* larvae used as template, 5 µl of 2m M dNTPs, 2 µl of 50Mm Mgcl2, 5 µl of PCR buffer, 4 µl of 10 µM primer, 1 µl of 5U/µl Taq. Polymerase and 27 µl of distal water). Temperature cycling was carried out by Perkin Elmer thermal cycler 2400 with 15 min at 96Cº and then 30 cycles of the following profile: 40 sec at 96Cº, 40 sec at 36Cº, and 2min at 72 Cº. After the 30 cycles the reaction were held at 72 Cº for 7 min. The PCR amplified gene products were digested with the endonucleases *Spe*I, *EcoR*I and *Bbs*I (New England Biolabs) to cut down the gene to detect the mutations induced by the different sub-lethal doses of the used IGRs. Samples were incubated overnight at 37 Cº. The restriction enzyme profiles were visualized with

ethidium bromide on 1.5% agarose gel and photographed under UV light.

Gene and Bioinformatical analysis

All the details of the normal *S. littoralis* vira-like chitinase gene such as origin, translation, percentage of bases, codons and amino acids …etc are explored by the (Bioface and DNA counter programs). The alignments of the vira-like chitinase gene sequences, the encoded amino acids and protein charges for the studied groups were obtained by (CLC Main Workbench program version 5.5). RNA secondary structure, stems nucleotide sequences, positions and free energy were recorded as well as the distinct secondary structure was drawn as well as the phylogenetic tree using a computer program known as gene bee RNA secondary structure prediction (Genebee service,http://www.genebee.msu.ru/cgi-bin/nph-malign.pl).

RESULTS

Biological Studies:

Results recorded in Table (1) showed that lufenuron had a significant effect on the larval and pupal duration as the mortality increase with the increase of the concentration. The larval mortality increased to 100 % at concentration 2ppm while pupal mortality was 11.3 % at 1.5 ppm of lufenuron. The larval and pupal durations showed significant increase with the increase of concentration. Specially for pupae which showed very high increase elongation at doses 0.5, 1.0 and 1.5 ppm (10, 11 and 13 days, respectively) compared with control (7 days). On the other hand, the pupation percentages were greatly reduced to 90, 60, 50 and 20 % at the concentrations of lufenuron 0.1, 0.5, 1.0, 1.5 and 2.0 ppm, respectively. The percentage of the adult emergence was decreased with the increase of concentrations as indicated by 85, 52.5, 40 and 8.75 %, respectively, at ascending successive concentrations. The present results indicated that there was a significant inhibition of adult emergence percentages as 15, 47.5, 60, 91.25 and 100 % as compared with control 0.0 %.

Table 1: Effect of lufenuron on some biological aspects of the cotton leafworm *S. littoralis* by feeding newly 2nd instar larvae on treated castor leaves for 24hrs.

Conc. (ppm)	Larval mortality % ±S.E	Larval duration (days) ±S.E	Pupation % ±S.E	Pupal mortality % ±S.E	Pupal duration (days) ±S.E.	Emerged moths % ±S.E	Total inhibition of adult emergence %
0.0	0	10±0.41	100±0.0	0	7±0.41	100±0.0	0
0.1	** 10±0.0	10±0.41	**90±0.0	5±0.0	8.5 ±0.29	***85±0.0	***15±0.0
0.5	***40±0.41	11±0.58	***60±0.41	**7.5±0.29	***10 ±0.41	***52.5±0.5	***47.5±2.5
1.0	***50±0.58	12±0.58	***50±0.58	***10±0.41	***11±0.58	***40±0.41	***60±2.04
1.5	***80±0.41	* 13 ±0.41	***20±0.41	***11.3±05	***13±0.41	***8.8±0.25	***91.3±1.3
2.0	***100±0.0	8 ±0.41	0	0	0	0	***100±0.0

*Significant at P = 0.05   ** High significant at P = 0.01 *** Very high significant at P = 0.001
+Ovipositiondeterrenet index.

The results present in Table (2) showed that lufenuron induced a reduction in both fecundity and fertility, on the contrast (O.D.I) and sterility showed an positive relationship with the increase of concentrations. The mean

number of eggs deposited by female resulted from treated larval instar with lufenuron were lower significantly than the check. Average numbers of laid eggs were 1385, 1275, 1156 and 945eggs/female, respectively. While the mean number of eggs deposited by untreated female were 1385eggs/female.

The percent of egg hatching was high significant affected by treatments. The hatchability percentages of eggs were 98.9, 94.02, 90.08 and 85.18%, respectively for females emerged from treated by 0.1, 0.5, 1.0 and 1.5ppm, respectively, compared with 100% eggs/ females of control.

Table 2: Effect of lufenuron on fecundity, fertility and sterility against adults of cotton leafworm *S.*

Conc. (ppm)	No. of eggs/female (fecundity) ±S.E	+O.D.I % ±S.E	Egg hatching (fertility) % ±S.E	Sterility % ±S.E
0.0	1385±30.48	0.0	100±0.0	0.0
0.1	1385±30.48	2.55±0.73	98.9±0.42	4.5±1.42
0.5	1275±30.62	4.13±1.51	*** 94.02 ±1.06	***13.4±2.28
1.0	***1156±32.19	***9.01±2.4	***90.08 ±0.43	***24.6±3.39
1.5	***945±20.61	***18.88 ±0.49	***85.18 ±1.49	***41.7±1.17
2.0	***0.0	0.0	*** 0.0	0

littoralis emerged from 2nd larval instar feeding on treated castor leaves.

*Significant at P = 0.05   ** High significant at P = 0.01 *** Very high significant at P = 0.001
  +Ovipositiondeterrenet index.

Toxicological Studies:

The Susceptibility of the 2nd larval instars of *S. littoralis* to lufenuron was showed in Table (3). The corresponding concentrations LC_{25}, LC_{50} and LC_{90} were 0.3, 0.6 and 2.5 ppm, respectively.

Morphogenic abnormalities and malformation shapes:-

The morphogenic abnormalities of larvae, pupae and adults which emerged from 2nd larval instars treated with the tested IGR could be grouped into four categories: malformed larval instar, larval-pupal intermediates, malformed pupae and malformed adults.

Table 3: Toxicological evaluation of lufenuron against 2nd larval instars of *S. littoralis*

Larval instar	Toxicity of match ppm			Slop function
	LC_{25}	LC_{50}	LC_{90}	
2nd instar	0.3	0.6	2.5	2.1

Molecular studies:

In the present study the sequence of the *S. littoralis* vira-like chitinase gene, 1218 pb, 398 encoded amino acids and 8 stop codons was used to determine the predicted mutations as a result of lufenuron treated 2nd instar larvae.

The digestion of vira-like chitinase gene of the different doses of lufenuron at the different time intervals post treatment with the endonuclease *Spe*I resulted in two fragments of 137 and 1081 bp in length (plate 1).

*Eco*RI endonuclease digests the gene of the treated larvae with LC_{90} of lufenuron after 6hrs post treatment (plate 2) into three fragments of 129, 334 and 755 bp in length (lane 4). Remaining different treated groups (LC_{25} and LC_{50}) have the similar restricted fragments as normal with lengths 334pb and 884pb (lane 2 and 3, respectively). Plate (3) showed that the endonuclease enzyme *Eco*RI digests the gene at sub lethal doses LC_{50} and LC_{90} after 12hrs, lanes (3-4) resulted in three bands with lengths 129

pb, 334pb and 755pb. LC$_{25}$ treated groups has the similar restricted fragments as normal with lengths 334pb and 884pb. After 24&48hrs post treatment, *EcoRI*cut cut the gene in three restricted fragments (plate 4). These restricted fragments are equal in the different groups with lengths129pb, 334pb and 755pb.

The enzyme *Bbs*I cut the vira-like chitinase gene of the treated larvae with sub lethal dose LC$_{90}$ after 6hrs post treatment (plate 5) producing three bands (lane 4) these bands with lengths 60 pb, 134 pb and 1024 pb. The Remaining groups as well as normal have the same lengths 60 pb and 1158 pb.The restricted fragments, which resulted from the digestion of vira-likechitinase gene with the enzyme *Bbs*I after 12hrs post treatment showed in plate (6). Three distinct bands in lanes (3,4) at lengths 60 pb,134 pb and 1024 pb as a result of treated larvae with the sub lethal doses LC$_{50}$ and LC$_{90}$ of lufenuron, respectively. Digestion of Vira-like chitinase gene with the enzyme *Bbs*I showed in plate (7). In lane 2 there were three bands at

lengths 60 pb, 517 pb and 641pb distinct to the sub lethal dose LC$_{25}$ of lufenuron. The enzyme *BbsI* cut the gene of the treated larvae with sub lethal doses LC$_{50}$ and LC$_{90}$ after 24&4 8hrs post treatment represented in lanes (3-4, respectively) three times resulting in four bands with lengths 60 pb, 200 pb, 441pb and 517pb.

From all above results we can recognized that we have four groups of mutated vira-like chitinase gene resulted from the treatment of the 2nd larval instar with the different sub lethal doses of lufenuron at different times intervals post treatment (Table 4).

The 1st group (G$_1$) includes the treated larvae with, LC$_{25}$ after 6 &12 hrs as well as LC$_{50}$ after 6 hrs The 2nd group (G$_2$) included the treated larvae with LC$_{90}$ after 6hrs. The first mutation involved the substitution of the nitrogenous base T (No.194) with C; the second mutation involved the substitution of the nitrogenous base G (No.468) with C. Therefore according to these two mutations the encoded amino acids showed two mutations.

Table 4: Groups of mutated vira-like chitinase gene resulted from the treatments of the 2nd larval instar of *S. littoralis* with the different sub lethal doses of match at the different times intervals post treatments..

Treated groups	DNA sequence				
	Nucleotide change	Nucleotide change Position	Amino acid change	Amino acid position	Restriction enzymes
G1	——	——	——	——	*EcoRI* *BbsI*
G2	T ► C G ► C	194 468	I ► T L ► F	65 156	*EcoRI* *BbsI*
G3	G ► C T ► C	468 701	L ► F I ► T	156 234	*EcoRI* *BbsI*
G4	G ► C G ► C T ► C	260 468 701	R ► T L ► F ► T	87 156 234	*EcoRI* *BbsI*

The first one involved the substitution of the amino acid I (No.65) with T, the second involved the substitution of the amino acid L (No.156) with F. Exposed 2nd larval instar to LC$_{50}$ and LC$_{90}$ of lufenuron after 12hrs post

treatment as well as LC$_{25}$ after 24&48 hrs showed two mutations(G3). The first one involved the substitution of the nitrogenous base G (No.468) with C, the second mutation involved the substitution of the nitrogenous base T (No.701) with

C. Therefore according to these two mutations the encoded amino acids showed two mutations. The first one involved the substitution of the amino acid L (No.156) with F, the second involved the substitution of the amino acid I (No.234) with T.

The treated 2nd larval instar with LC$_{50}$ and LC$_{90}$ of lufenuron showed three mutations after 24&48hrs post treatment (G4). The first one involved the substitution of the nitrogenous base G (No.260) with C, the second mutation involved the substitution of the nitrogenous base G (No.468) with C, the third mutation involved the substitution of the nitrogenous base T (No.701) with C. Therefore according to these three mutations the encoded amino acids showed three mutations. The first one involved the substitution of the amino acid R (No.87) with T, the second

involved the substitution of the amino acid L (No.156) with F, the third mutation involved the substitution of the amino acid I (No.234) with T.

RNA which transcribed from vira-like chitinase gene is a single strand, this strand has several complementary sequences (paired) stems and single stranded (unpaired) loops. The stems with complementary nucleotides and resulted loops formed a distinct structure known as RNA secondary structure. As the yielding protein of the mutated vira-like chitinase gene has been changed, the RNA secondary structure also is expected to be changed. The number of RNA secondary structure stems is varied in the different studied groups and most of them differed in their nucleotide sequences, positions and free energy Table (5).

Table 5: Free energies of the predicted RNA structure of the different studied groups of *S. littoralis* larvae treated with lufenuron.

Groups*					
	N	G1	G2	G3	G4
total number of stems	40	40	39	39	38
Free energy Kkal/mol	-202.7	-202.7	-194.8	-192.4	-184.1

N: normal insects.

G1: LC$_{25}$ treated larvae after 6 and 12 hrs as well as LC$_{50}$ treated larvae after 6hrs.

G2: LC$_{90}$ treated larvae after 6hrs.

G3: LC$_{50}$ and LC$_{90}$ treated larvae after 12hrs as well as LC$_{25}$ after 24&48 hrs.

G4: LC$_{50}$ and LC$_{90}$ treated larvae after 24&48hrs.

For each studied group, the RNA secondary structure stems nucleotide sequences, positions and free energy were recorded as well as the distinct secondary structure was drawn using a computer based online program known as gene bee RNA secondary structure prediction (Figs.1-4).
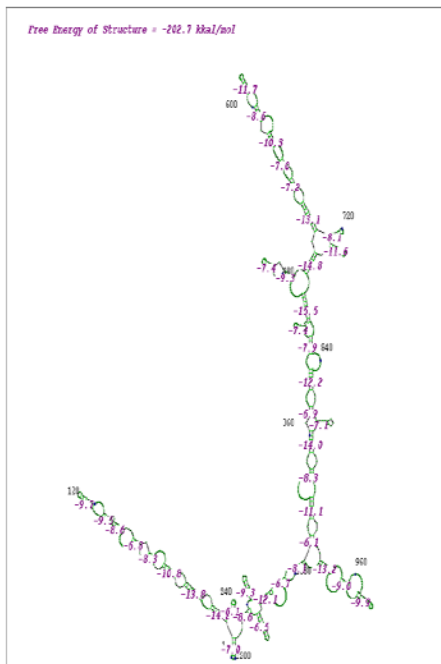
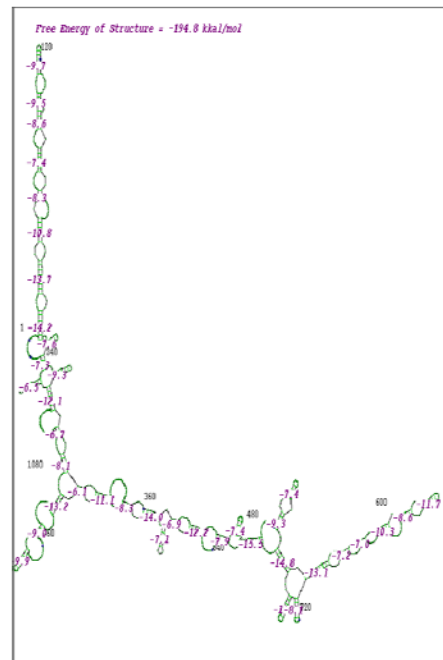Fig. 1: Shows the structure of the predicted RNA structure of the normal and 1st group.

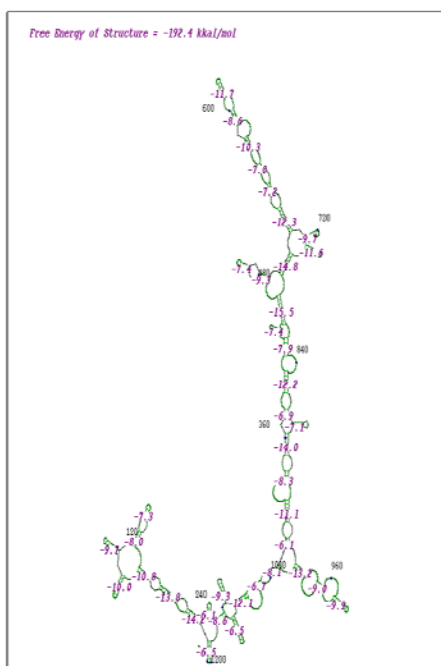Fig. 2: Shows the structure of the predicted RNA structure of 2nd group.

Fig. 3: Shows the structure of the predicted RNA structure of 3rd group.
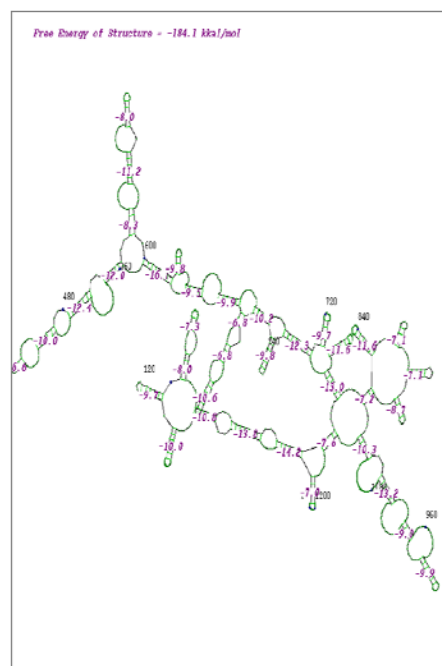

Fig. 4: Shows the structure of the predicted RNA structure of 4th group.

DISCUSSION

In this work, the chitin synthesis inhibitor lufenuron showed significant control on *S. littoralis*. Susceptibility test on a laboratory strain of 2nd instar larvae of cotton leafworm indicates that there were positive correlations with the tested concentrations.   The mortality in the larval stage was clearly due to the moulting disrupting effect of the lufenuron. This effect is mainly induced by inhibiting chitin formation (Abdel Rahman *et al*., 2007), thereby causing abnormal endocuticular deposition and abortive moulting (Mulder and Gijswigt, 1973). Furthermore higher concentrations have antifeeding effect. Therefore, feeding impairment of treated larvae could lead to prolongation of the larval instars and subsequently leading to a reduction in the percentage of pupation and adult emergence.   Gelbic *et al.* (2011) showed that larval instars of *S. littoralis* treated with lufenuron unable to complete the molting process and died in the old larval cuticle, likewise, flufenoxuron caused similar effect on *S. littoralis* treated larvae (Baker *et al*., 2010).   Pupal mortalities in this study were obvious; there were dose-dependent effect on pupation and pupal mortalities. The same results were found by (Butter *et al*., 2003; Biddinger *et al*., 2006 and Salokhe *et al*., 2008).

It was obvious that the percent of inhibition of adult emergence were in positive relation with the increase of concentrations. These results was also found by (Osman & Mahmoud 2009) who demonstrated that the emergence of Egyptian cotton leafworm moth affected by treatments larvae with lufenuron compared to the control. Also the treatment with lufenuronat significantly reduced the reproductive potential of *S. littoralis*. The reduction in fecundity and egg hatchability of the cotton leafworm following treated with this IGR was also observed by Abdel Rahman *et al*., (2007) and Adel (2012).

Reduction in fecundity may be due to the reduction in longevity and the number of oocytes per ovary and the reduction in oviposition period (Soltani and Mazouni, 1992). In addition to the above factors the maturation of an insect egg depends on the materials that are taken up from the surrounding haemolemph and materials synthesized by the ovary. These materials include protein, lipids and carbohydrates all of which required for embryonic structure (Kanost *et al*., 1990). Subsequently the reduction in total number of eggs per female could be due to interference of the IGR with oogenesis.   They induce decrease in the concentration of yolk proteins, carbohydrates, lipids and inhibition in both DNA and RNA synthesis in the ovaries of females treated as larval instars, moreover they caused vacuolation of nurse cells and oocytes of the ovaries (Shaurub *et al*., 1998).

Reduction in the percentage of egg-hatch obtained in the present study may be due to defects in the differentiation of oocytes and sperms (Meola& Mayer 1980 and Horowitz *et al*., 1992). Emam and Degheel (1993) found that treating 4th instar larvae of *S. littoralis* with sublethal doses of chlorfluazuron and diflubenzuron induced decrease in adult emergence from 80% in control to 14 and 23%, respectively, also viability of deposited eggs and progeny formation was reduced. For explanation, Sallam (1999) indicated that ovicidal activity of flufenoxuron in eggs deposited by *S. littoralis* that emerged from treated larvae could be due to disturbance in cuticle formation of the embryo, developed embryos were not able to perforate the surrounding vitelline membrane, it could be due to a weakened chitinous mouth parts that was insufficiently rigid to affect hatching. Morphogenic   abrriations   and

abnormalities in larval, pupal and adult stages were recorded in this study, as well as intermediates of larval-pupal and pupal adult forms, these findings are in agreement with those results obtained by (Whiting *et al.*, 2000 ; Butter *et al.*, 2003 ; Mourad *et al.*, 2006; Salokhe *et al.*, 2008).

In the present study, the molecular observations and bioinformatics results showed a different mutation in the vira-like chitinase gene depending on the dose of lufenuron and the time post-treatment. These mutations increased with increasing in both dose and time post-treatment and in change in RNA secondary structure. According to these changes the minimum free energy and number of stems were changed. A viral-like chitinase was found to be up-regulated. The degradation of cuticular chitin by chitinases is a vital step prior to ecdysis and metamorphosis (Kim *et al.*, 1998). Kramer *et al.*, 1993) reported that the chitinase gene of *Manduca sexta*was most highly expressed in epidermal and gut tissues during the larval-pupal metamorphosis and its transcription was stimulated by ecdysterone (20E) and inhibited by a JH mimic. The chitinase might be involved in remodelling of the integument during metamorphosis.

RNA plays many diverse roles in biology, including catalyzing peptide bond formation) Hansen *et al.*, 2002), catalyzing RNA splicing (Doudna and Cech 2002), localizing protein and flagging development (Lagos-Quintana *et al.*, 2001).

The stability of the RNA secondary structure is quantified as the amount of free energy being released or used by the forming base pairs. The stability increases according to the number of GC versus AU and GU base pairs and the number of base pairs in a hairpin loop region. The number of unpaired bases decreases the stability of the structure such as interior loops, hairpin loop or bulges (Mohsen *et al.*, 2009).The

stability of the secondary structure depends on the amount of free energy released to form the base pairs. Thus, the more negative the free energy of a structure is, the more stable a particular sequence is formed. This structure is called the MFE secondary structure (Layton and Bundschuh 2005).

Minimum free energy of the secondary structure represents the amount of energy that could be needed for the ribosomal RNA to translate the RNA structure into protein, the highest minimum free energy of the structure needs more energetic ribosomal RNA to translate it, also number of stems may be play a role in rate of translation, the more number of stems of the structure the more time needed to translation.

The above mentioned facts about the secondary structure may be represent a molecular explanation to the malformations appears in the present study as well as other physiological changes, as enzymatic activities and embryogenesis process.

Many researchers observed that DNA biosynthesis was inhibited in several insects' species as a result of treatments by CSIs, so inhibition of DNA synthesis would explain most of CSIs effect on insects.

Meola and Mayer (1980) studied the effect of the Diflurodenzuron on the pharate pupa of *Stomoxy scalcitrans*. They found that the formation of the adult imaginal epidermis was inhibited as a result of DNA biosynthesis inhibition. Soltani *et al.*, (1984) investigate the effect of Diflurodenzuron on the pupae of *T. molitor*. They observed that the DNA biosynthesis as well as mitosis in the epidermis of the abdominal sternites was affected as a result to this chitin synthesis inhibitor. Assar and Emara (1997) tested the histochemical effect of Dimilin on the midgut and integument of the 4th larval instar of *S. exigua*. They found a high decrease of DNA in nuclei of the midgut cells and slight decrease of RNA in

cytoplasm of hypodermal cells. Shaurub *et al*., (1998) reported that the IGRs, Pyriproxyfen and diethyl ether leaves extract of the Brazilian pepper, *Schinus terebinthifolius*, reduced the synthesis of RNA and DNA in *S. littoralis* ovarioles. Histochemical studies on the testes showed that RNA, DNA and protein synthesis were inhibited during spermatogenesis. The mutagenic potentiality of lufenuron to *Drosophila melanogaster* was assessed by Abd-Alla *et al*., (2003). They observed that lufenuron induced changes in the genomic DNA of the whole body of lufenuron-treated *D. melanogaster*, that estimated by the occurring DNA polymorphism. The treatments have resulted in emergence of some adults with folded wings of rough texture. These abnormal adults lost the flight ability in both the parents and the second generation.

Because of the crucial roles of chitinases in insect growth and development, these enzymes have been widely recognized as potential targets for developing chemical pesticides for insect control (Hirose *et al*., 2010).

In conclusion, our study on the treatment of the cotton leafworm *S. littoralis* with lufenuron revealed expected toxic and biological effects on larva, pupa and adult stages. Also the endonucleases *Spel, EcoRI* and *Bbsl* were used to detect the molecular changes in the vira-like chitinase gene in the different studied groups. Bioinformatical studies showed a significant changes in the sequences of the treated groups (mutations) which were dose and time dependants.
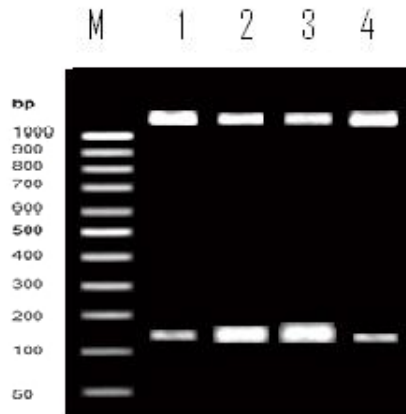
Plate 1: Represents that *SpeI* restricted the vira-like chitinase gene of all groups at the different time intervals post treatment with the same lengths 137pb and 1081pb.
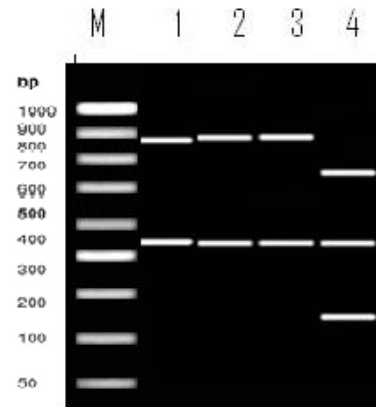
Plate 2: Shows the digestion of enzyme *EcoRI* to the vira-like chitinase gene of all groups at 6hrs. post treatment.
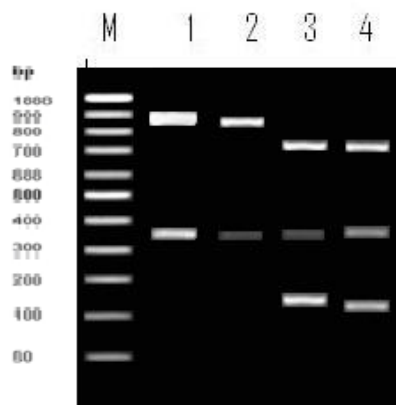
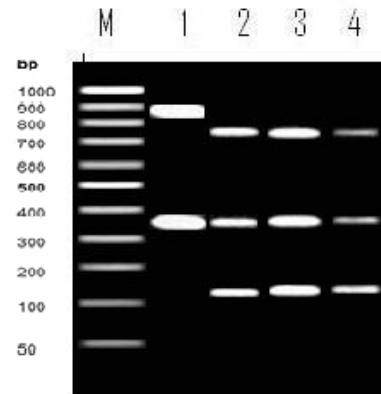Plate 3: Shows the digestion of enzyme *EcoRI* to the vira-like chitinase gene of all groups at 12hrs post treatment

Plate 4: Shows the digestion of enzyme *EcoRI* to the vira-like chitinase gene of all groups at 24 &48 hrs post treatment.
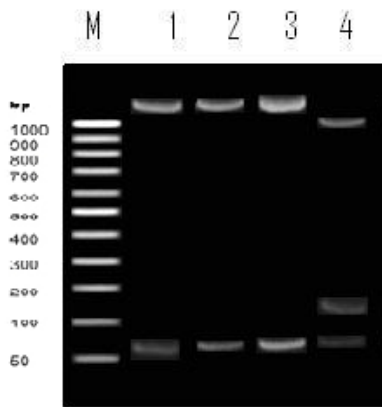
Plate 5: Shows the digestion of enzyme *BbsI* to the vira-like chitinase gene of all groups at 6 hrs post treatment.
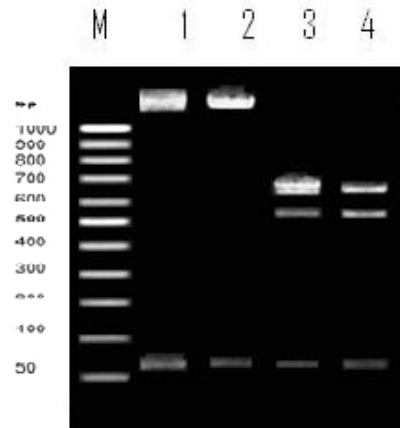

Plate 6: Shows the digestion of enzyme *BbsI* to the vira-like chitinase gene of all groups at 12 hrs post treatment
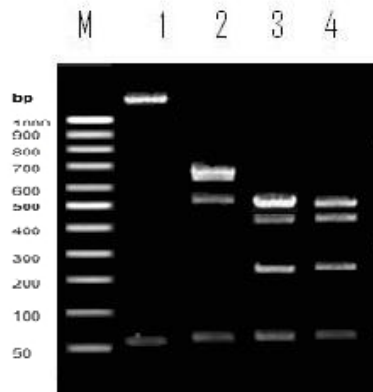

Plate 7: Shows the digestion of enzyme *BbsI* to the vira-like chitinase gene of all groups at 24&48 hrs post treatment.

M : Marker   **1** : Normal   **2**: LC_{25} of Match
3: LC_{50} of Match   **4**: LC_{90} of Match

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