Chitinase gene mutations induced by two insect growth regulators in *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract: This piece of work aims to evaluate the oral-uptake sub-lethal doses LC_{50} & LC_{90} against molting hormone agonist, ecdysone agonist, (methoxyfenozide) and chitin synthesis inhibitors, benzoylphenylurea derivatives, (triflumuron) on *Spodoptera littoralis* biology and chitodextrinase enzymegene. These takes place by catalyzing the random hydrolysis of N-acetyl-beta-D-glucosaminide (1->4)-beta-linkages in chitin and chitodextrins as IGRs effect chitin formation and degardation. The obtained results demonstrated that triflumuron was more powerful against 2nd larval instar, with LC_{50} and LC_{90} (0.4 and 1.5 mg/L) respectively, than methoxyfenozide which has LC_{50} and LC_{90} (1.625 and 2.625 mg/L) respectively, on all biological aspects (fecundity, fertility), also by inducing many morphological abnormalities. Induced mutations by using both sub-lethal doses LC_{50} & LC_{90} were screened in the transcribed mRNA and the predicted proteins after two days of treatments. Chitin synthesis inhibitor was more effective than molting agonist of *Spodoptera littoralis* biology and chitodextrinase enzymegene. The present study highlights the mode of action of IGRs during the process of chitin formation and degradation in cotton leafworm (*Spodoptera littoralis*) larvae as a target and model insect.
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1.Introduction

The Egyptian cotton leaf worm, *Spodoptera littoralis* Bosid, (Lepidoptera: Noctuidae) is an important pest of cotton in Africa, Middle East and Southern Europe larvae feed mainly on leaves, stems and also can seriously retard growth or reduce production of the cotton crop. However, during heavy infestation they can penetrate flowers and bolls **(Sutanto *et al*., 2014)**. Many control methods were carried out to suppress the pest population and keep it under the economic injury level. The recent intensive research is concerned mainly with avoiding the serious problems resulted from using harmful insecticides that cause harmful residues in the food chain and pollution of the surrounding natural enemies and pest resistance **(El-Mageed *et al*., 2014)** . The necessity to find environmentally safe insecticides as well as materials to combat species resistant to conventional pesticides has spurred increased interest in alternative insecticides such as utilization of plant extraction and insect growth regulators (IGRs) **(Ahmed *et al*., 2014)** . Insect growth regulators, a third generation of chemical, effective in insect control arose from basic studies of insect endocrinology and biochemistry. Insect hormones are internal secretions which regulate a wide range of physiological processes, including growth, development and maturation, they are currently evaluated for insecticidal efficacy, IGRs are considered to have little human toxicity, the use of IGRs compounds in insect control is known as insect developmental inhibition, which inhibits or prevents normal metamorphosis of immature stages to the adult stage. These compounds have been tested successfully against several insect species **(Bouzeraa *et al*., 2014)**.

Chitin is one of the most important biopolymers in nature; it is mainly produced by fungi, arthropods and nematodes. In insects, it is playing a canonical key role for supporting the cuticles of the epidermis, trachea, peritrophic membrane and lining the gut epithelium as scaffolding material **(Qu *et al*., 2014)**. Insect growth and morphogenesis are strictly dependent on the capability to rebuild chitin containing structures, thus, insects repeatedly produce chitin synthases and Chitodextrinase enzymes in different tissues. Chitin synthesis and degradation requires strict control of the participating enzymes during development **(Muthukrishnan *et al*., 2012)**. 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 (**Doucet *et al*., 2012)**. Insect's chitinases homologues genes have been identified and characterized from several insects including lepidopteran species **(Kramer *et al*., 1993; *Kim et al*.,**

1998; Shinoda *et al*., 2001; Zheng *et al*., 2002; Bolognesi *et al*., 2005; Tyne & Possee 2005 and Zhou *et al*., 2014). Chitin synthesis inhibitors (CSI) block the production and formation of chitin. Because moulting must take place for the insect to reach the adult stage, a CSI poisoned insect can't make chitin, moult and can't reproduce thus eventually dies **(Kale; 2014)**. Moulting hormone agonists induce precocious and incomplete moults in different insect orders with various morphogenetic types, moulting hormone agonists, Ecdysone Like 20E, ply a major role via interaction with the ecdysteroid receptor complex, a heterodimer of the ecdysteroid receptor and ultraspiracle proteins **(Hill *et al*; 2013)**. Methoxyfenozide has been shown to act in a similar fashion as the endogenous ecdysteroid, 20E, in initiating the moulting process in lepidopteran insects (**Henrich; 2011)**. This work aimed to assess the effects of chitin synthesis inhibitor Benzoylphenylurea derivatives, Triflumuron and moulting hormone agonist, Ecdysone agonist, Methoxyfenozide on some biological aspects of *S. littoralis*, and sub lethal doses LC_{50} and LC_{90} effects on the Chitodextrinase enzymegene (identified from The European nucleotide archive and NCBI-http://www.uniprot.org/uniprot/Q3LS03) two days post-treatments. Obtained results highlighted the mode of action of IGRs and support the hypothesis that IGRs play an important role in the process of chitin formation and degradation in cotton leafworm (*Spodoptera littoralis*) larvae as a target and model insect by altering the transcribed mRNA.

2. Material and Methods

Insect culture

Insect culture was initiated from freshly collected egg masses supplied from Plant Protection Research Institute, Dokki, Egypt, larvae reared on castor leaves and maintained under laboratory conditions of 27±2 Cº and R.H. 70±5 %.

Oral-delivery of the tested IGRs solutions to larvae

Five concentrations were prepared by dissolving the tested IGRs in distilled water, Pieces of castor been leaves, were treated by leaf-dipping technique and left in air for 1h to insure completely dryness, eighty of starved larvae, distributed in five replicates for each concentration allowed to feed for 48 hrs on treated leaves, control larvae were allowed to feed on castor bean leaves that dipped only in distilled water. Final mortality percentages of the treated and control larvae were recorded 48 hrs post-treatment.

Screening of fecundity, fertility and strility in the emerged adults

Adult fecundity and sterility were screened by placing one female and one male together in a glass jar with a piece of cotton soaked in 10% sugar solution (source of food for moths) and covered internally with a soft sheet of paper for oviposition, egg patches counted daily, three patches having not less than 100 eggs were collected during the first 3 days of oviposition and incubated until hatching, the percentage of hatchability was recorded and was used to calculate the percent of sterility.

RNA isolation and cDNA synthesis

Total RNA was isolated from pools of larvae (4 larvae), using TRIzolR® Plus RNA Purification Kit (Ambion, TRI reagent), purity and quality of RNA samples were evaluated and quantified spectrophotometrically. cDNA synthesis was performed by using the i-script™ reverse transcription supermix for RT-qPCR kit, 1000 ng of the extracted total RNA were used for reverse transcription in a final reaction mixture volume of 20µl, the reaction was mixed carefully and placed into the thermal cycler following the protocol.

Polymerase Chain Reaction and Cloning

PCR reactions were performed in (Applied Biosystems, GeneAmp PCR system 2700), PCRs were performed in 50µl reaction volumes using (Thermo scientific PCR master mix (2x). The final concentration of reagents used for the majority of PCRs was as follows: dNTP 0.4mM, Magnesium Chloride 4mM, primer concentrations 0.2Mm (F.P_ GTAAAGGAGATTGAAGGCAGTTTC, R.P_ TCACGCATTGTTACCGAGACCCAT), taq DNA polymerase, 1.25 Units/reaction. The cycling parameters were as follows: Denaturation step at 95^0C for 3 minutes, followed by a cycle of denaturation at 95^0C for 30s, annealing at 57^0C for 30s and elongation step at 72^0C for 75s. This cycle was repeated 35 times followed by a final elongation cycle of 15 minutes. PCR products were detected by gel electrophoresis through 1% agarose gel in 1xTBE with 0.5µg/ml Ethidium Bromide at 85V for 1hr. The PCR products compared to 100pb DNA ladder (Life technologies). PCR reaction bands were gel purified QIAquick MinElute Gel Extraction kit and stored at - 20^0C.

PCR products were ligated into StrataClone vector pSC-A by pipetting 3µl cloning buffer, 2 µl of gel purified PCR product and 1 µl of StrataClone vector mix amp/kan, incubated for 5 minutes at room temperature then placed on ice. 1 µl of the above cloning mixture was added to StrataClone SoloPack competent cells and mixed gently, and incubated on ice for 20 minutes followed by heat shock at 42C for 45 seconds and again on ice for 2 minutes. Pre-warmed 250 µl of S.O.C. medium were added to the Transformation mixture and incubated in37C for 2 hours with agitation before plating on Ampicillin plates, 50 µl and 100 µl of the Transformation mixture were plated on LB-Ampicillin agar plates that have been spreaded with 40 µl of 2%X-gal and incubated

for overnight at 37C. Blue/white screening and performing colony were picked and cultured overnight in L.B Broth at37C with agitation. Bacterial cells were pelleted from LB media by spinning at 6000xg for 4 minutes. Plasmid DNA was extracted by using a QIAprep spin Miniprep kit, Verification of cloned fragments was performed by dideoxy sequencing of products using M13 primers, sequencing performed by commercial organisation.

Calculations and Statistical analysis

Percentages of larval mortality were corrected according to Abott's formula **(Abbott; 1925)**, the percent of adult emergence inhibition, toxicity index, oviposition deterrenet index (O.D.I) and fertility were calculated according to **(Jahan *et al.,* 2014; Ghoneim; *et al.*,2012; Senrung *et al.*,2014 and Salem *et al.*,2014)**, the oviposition deterrenet index (O.D.I) was considered as indicator of female fecundity. Experimental data results were analyzed by Student's t-Test and analysis of variance and the statistical significance are shown as different letters. T-Coffee Multiple Sequence Alignment Tools **(Di Tommaso *et al.*,2011)** was used to generate alignments between the obtained chromatograms from sequencing to detect the resulted mutations in the transcribed mRNA.

3.Results

Oral toxicity and dose response curves

Toxicological activity of the tested IGRs are summarized in **Table (1)**, the tested IGRs could be arranged descendingly according to their potency against 2nd larval instar of *S. littoralis* at the bases of the LC_{50} and LC_{90} levels as following: Triflumuron followed by Methoxyfenozide, the corresponding concentrations of LC_{50}were: 0.4 and 1.625 mg/L respectively and The corresponding concentrations of LC_{90}were: 1.5 and 2.625 mg/L respectively, whereas the toxicity lines are drawn in **Fig. (1) and Fig. (2)**. Triflumuron exhibited the highest toxic action against 2nd larval instar followed by Methoxyfenozide in toxicity. Obtained results in **Table (1)** showed that the toxicity index at LC_{50} and LC_{90} levels.

Effect of dietary Introduction of treated Castor Leaves for 48hrs on some biological aspects of the cotton leaf worm

Corrected percentages of larval mortality had a direct correlation with the sub-lethal doses of Triflumuron, the percentages of mortality were 48 and 85 % after treatment with LC_{50} and LC_{90} respectively, with a total inhibition of adult emergence 76 and 92.75 % against LC_{50} and LC_{90}, respectively Table (2) and Fig. (3). On the other hand the data obtained in Table (4) and Fig. (3) showed that the sub-lethal doses of Methoxyfenozide induced larval mortality with 48.75 and 89.25 % after treatment with LC_{50} and LC_{90}, respectively and a final total inhibition of adult emergence with 73 and 91.25 % against LC_{50} and LC_{90}, respectively. Fecundity and fertility have inverse correlation; the data obtained in Table (3) and Fig. (4) showed that O.D.I was 4.08 % and 15.3% after treatment with LC_{50} and LC_{90} of Triflumuron respectively, with a sterility 20.9 % and 68.3 % for LC_{50} and LC_{90} respectively. On the other hand the data obtained in Table (5) and Fig. (4) Illustrated that O.D.I was 3.65 % and 6.2% after treatment with LC_{50} and LC_{90} of Methoxyfenozide respectively, with sterility 14.8 % and 24.3 % for LC_{50} and LC_{90} respectively.

Morphogenic abnormalities

Treated 2nd larval instar with sub-lethal doses of Triflumuron and Methoxyfenozide showed some morphogenic abnormalities ranging from Shrinked larvae with greenish chitinization on thorax, larvae that failed to shed the exuvia of 2nd instar and Intermediate instar between 2nd and 3rd instars Fig. (5), on the other hand, pupal stage showed some developmental abnormalities ranging from Larval-pupal intermediates, C- shaped pupae with a ring of larval cuticle around the abdomen and pupae with enlarged and shortened body Fig. (6), in addition to some emerged adults have various degrees of morphogenic abnormalities, Pupal-adult intermediate, adults were unable to emerge from their pupal skins (failure adults' emergence), and adults were completely free but possessed crumpled and incomplete formation of wings Fig. (7).

Mutations screening in the transcribed mRNA and predicted proteins

Obtained chromatograms sequencing files used to detect mutations in the transcribed mRNA using T-Coffee Multiple Sequence Alignment Tools. Larvae fed on Triflumuron treated leaves with LC_{50} and LC_{90} for 48hrs showed five mutations in the transcribed mRNA Fig. (8), at position No. 194 (U) transcribed to (C), in positions No. 333 and 336 (G) transcribed to (C), at position No. 332 (G) transcribed to (U), at positions No. 466 (C) transcribed to (U) and in positions No. 468 (G) transcribed to (C), at positions No. 602 (A) transcribed to (U), at positions No. 730 (G) transcribed to (A), at positions No. 732 (C) transcribed to (G), at positions No. 949 (A) transcribed to (U) and at positions No. 951 (C) transcribed to (G),whilst, the predicted proteins showed a significant changes in the translated amino acids, at position No. 65 (I) replaced by (T) ,at position No. 111 (W) replaced by (F), at position No. 112 (E) replaced by (D), at position No. 156 (L) replaced by (F), at position No. 201 (Q) replaced by (L) and at position No. 244 (G) replaced by (R) and at position No. 317 (S) replaced by (W) deleted Fig. (9). On the other hand, larvae allowed feed on treated leaves with LC_{50} and LC_{90} with Methoxyfenozide for 48hrs showed

three mutations in the transcribed mRNA Fig. (8), at position No. 332 (G) transcribed to (U), at position No. 333 (G) transcribed to (C) and at position No. 466 (C) transcribed to (U), at position No. 468 (G) transcribed to (C), at position No. 701 (U) transcribed to (G) whilst, the predicted proteins showed a significant changes in the translated amino acids, at position No. 111 (W) replaced by (F) ,at position No. 156 (L) replaced by (F) and at position No. 234 (I) replaced by (S) Fig. (9). Distribution of the encoded amino acids was varying across the transcribed mRNA from the control group and treated with IGRs Fig. (10).

4.Discussion

The insect integument consists of a single layer of epidermal cells attached to a basement membrane, from which arises the cuticle. The cuticle consists of an outer thin, waxy, and water-resistant epicuticle, which is important for water retention, and the much thicker inner procuticle, which consists of two layers built from interwoven fibers of protein and the long-chain polysaccharide chitin, a polymer of N-acetylglucosamine, accounts for about 3–13% (w/w) of its dry weight **(Muthukrishnan *et al.*,2012)** it functions as scaffold material, supporting the cuticles of the epidermis and trachea as well as the peritrophic matrices lining the gut epithelium **(Merzendorfer *et al.*, 2012).** Insect growth progresses through stages: eggs hatch into an immature stage, and the insect may pass through several more immature stages before emerging as an adult, as the exoskeleton cannot expand, it must be shed and replaced with a larger one at each molt to the next stage. Some insecticides target processes unique to insects, such as the biosynthesis of chitin, a tough, semitransparent polysaccharide that is the main component of the insect's exoskeleton, key insecticide target groups include insect growth regulators.

The results presented in this context clearly show that rate of mortality depends on the concentration levels when 2nd larval instar allowed to feed on pieces of castor been leaves treated with LC_{50} and LC_{90} of Triflumuron and Methoxyfenozide, Interestingly, Chitin synthesis inhibitor (Triflumuron) was more toxic than moulting hormone agonist (Methoxyfenozide), these results are consistent with **(Sáenz-de-Cabezón *et al.*,2006; Tabozada *et al.*,2014; Hooshmandi; *et al.*,2014)**, chitin synthesis inhibitor involved in insect growth and development during molting and due to its lipophilic properties it can interfere with the exoskeleton chitin by contact **(Abbas; *et al.*,2014)**. Furthermore higher concentrations have antifeeding effect, obtained results also with agreement with **(Gelbic *et al.*,2011)** who study the effect of Lufenuron on the development and reproduction of *S. littoralis* in comparison study with RH-5992, the results indicate that the Lefenuron was more active than RH-5992, alimentary canal (fore and hind gut) of the treated larvae is the first position to be affected with this compound, as well as the mid gut (peritrophic membrane) chitinases seem to be involved in the formation, perforation and degredation of the midgut peritrophic matrix, which protect the gut epithelium from damaging **(Lemos *et al.*,2002; Zhai *et al.*, 2002)**, in contrast of chitin synthesis inhibitor moulting (ecdyson) agonist, interfere with the action of moulting hormone involved in insect growth and development during moulting only, due to the ecdyson agonists are steroids and more hydrophilic than lipophilic, they cannot penetrate the wax layer of the cuticle and so fare ineffective on exoskeleton chitin by contact **(Tunaz; 2004)**, so this substance need to be injected into the larval body in order to interfere with moulting process **(Klowden *et al.*, 2013)**. On the other hand, some morphogenic abnormalities in larval, larval-pupal and pupal stages, as well as pupal-adult intermediate and adult abnormalities were recorded for both of the used compounds in this study, generally, Triflumuron was more powerful than Methoxyfenozide for inducing morphogenic abnormalities **(Whiting *et al.*,2000; Butter *et al.*,2003; Salokhe *et al.*,2006;Hufn; 2014)**.

Tested IGR significantly reduce the percentages of fecundity and fertility of the eggs produced by the adult progeny, reduction in total number of eggs per female in this work could be due 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 and vacuolation of nurse cells and oocytes of the ovaries **(Perveen; 2010; Wei *et al.*,2013)**, moreover egg maturation depend on the materials that are taken up from the surrounding haemolemph and materials synthesized by the ovary in suit, these materials includes protein, lipids and carbohydrates all of which required for embryonic structure (**Kim *et al.*, 2011; Soltani-Mazouni *et al.*,2012)**, whereas, Ovicidal activity of the tested IGRs in the could be due to the disturbance in cuticle formation of the embryo and developed embryos were enabled to perforate the surrounding vitelline membrane, it could be due to a weakened chitinous mouth parts that was insufficiently rigid to effect hatching and ultimately lead to embryonic death **(Kostyukovsky& Trostanetsky 2006;Soltani-Mazouni *et al.*,2012)**.

Sanger dideoxy sequencing of PCR products was used in this investigation to analyze and scanning of the transcribed mRNA belonged to one of the Chitinases degredation genes in *Spodoptera littoralis* belong to family 18 glycosylhydrolases, which catalyse the Random hydrolysis of N-acetyl-beta-D-glucosaminide (1->4)-beta-linkages in chitin and

chitodextrins, after two days of treatments with sub-lethal doses LC_{50} & LC_{90} of the tested compounds. And it is clearly evident that chitin synthesis inhibitor (Triflumuron) induces mutations in the target gene at different positions and the predicted protein more than moulting hormone agonist, Ecdysone agonist, (Methoxyfenozide).

Table (1) Toxicity data of the tested IGRs against 2nd larval instar of *S. littoralis*

IGR	Conc. (mg/L)			
			Toxicity index	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Triflumuron	0.4	1.5	100	100
Methoxyfenozide	1.625	2.625	24.6	57.14

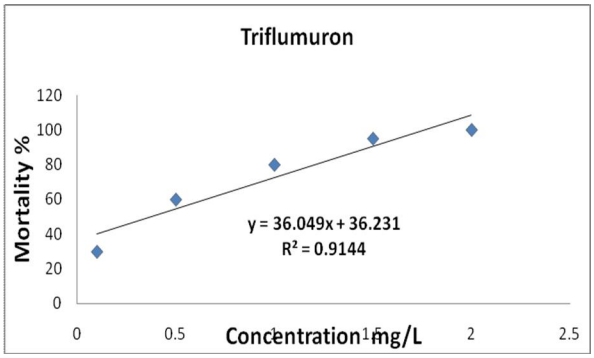

Fig. (1) Dose response curve of Triflumuron

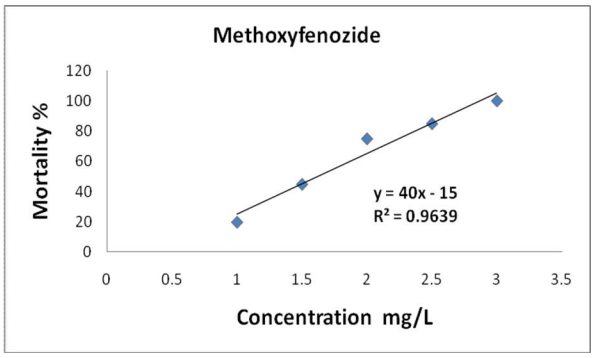

Fig. (2) Dose response curve of Methoxyfenozide

Table (2) Triflumuron bioassay against 2nd larval instar of *S. littoralis*

Conc. (mg/L)	O.D.I % mean±S.E	Sterility % Mean ±S.E
0.0	[a] ----- ±0.0	[a] ----- ±0.0
0.4	[b]4.08 ±0.9	[b]20.9 ±1.5
1.5	[c]15.3 ±1.1	[c] 68.3 ±3.8

Table (4) Methoxyfenozide bioassay against 2nd larval instar of *S. littoralis*.

Conc. (mg/L)	O.D.I % mean±S.E	Sterility % mean±S.E
0.0	[a] ----- ±0.0	[a] ----- ±0.0
0.4	[b]4.08 ±0.9	[b]20.9 ±1.5
1.5	[c]15.3 ±1.1	[c] 68.3 ±3.8

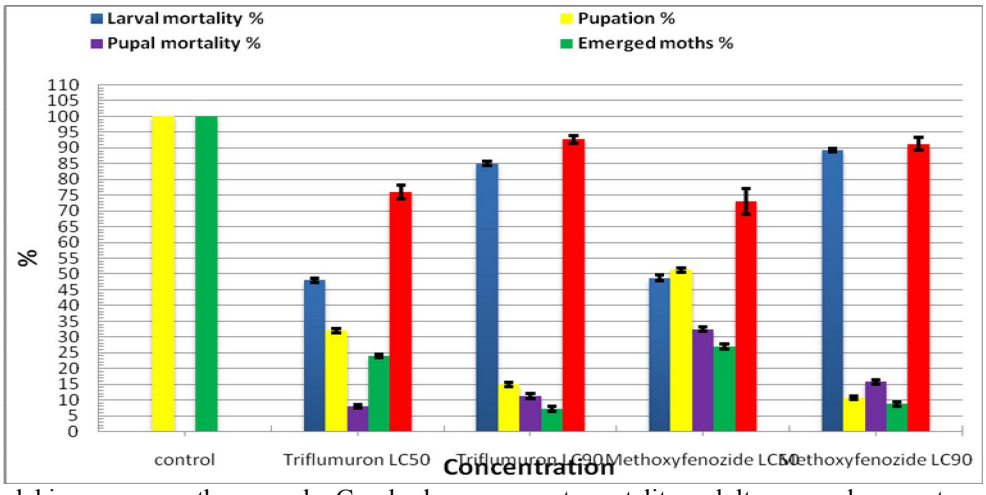

Fig. (3)Larval bioassay over three week. Graph shows percent mortality, adult emerged percent and total adult emergence inhibition. (4 biological replicates) means ± SE (Student's t-test, p= 0.001).

Table (3) Triflumuron effect on fecundity and sterility of the emerged adults.

Conc. (mg/L)	Larval mortality %mean ±S.E	Larval duration (days)mean ±S.E	Pupation %mean ±S.E	Pupal mortality %mean ±S.E	Pupal duration (days)mean ±S.E.	Emerged moths %mean ±S.E	Total inhibition of adult emergence %
0.0	[a]0.0 ±0.0	[a] 10 ±0.41	[a] 100 ±0.0	[a] 0.0 ±0.0	[a] 7 ±0.41	[a] 100 ±0.0	0.0 ±0.0
0.4	[b]48 ±0.71	[b]11.75 ±0.85	[b]32 ±0.71	[b]8 ±0.65	[b]12 ±0.41	[b]24 ±0.55	[b]76 ±2.04
1.5	[c]85 ±0.66	[c] 13 ±0.91	[c]15 ±0.64	[c]11.25 ±0.75	[c]14 ±0.56	[c]7.25 ±0.85	[c] 92.75 ±1.25

Table (5) Methoxyfenozide effect on fecundity and sterility of the emerged adults.

Conc. (mg/L)	Larval mortality %mean ±S.E	Larval duration (days)mean ±S.E	Pupation %mean ±S.E	Pupal mortality %mean ±S.E	Pupal duration (days)mean ±S.E.	Emerged moths %mean ±S.E	Total inhibition of adult emergence %
0.0	0.0 ±0.0	[a] 10 ±0.41	[a] 100 ±0.0	0.0 ±0.0	[a] 7 ±0.41	[a] 100 ±0.0	[a]0.0 ±0.0
1.625	[d]48.75 ±0.91	[d] 13 ±0.44	[d]51.25 ±0.61	[d]32.5 ±0.78	[d]10 ±0.41	[d]27 ±0.82	[d] 73 ±4.08
2.625	[e]89.25 ±0.51	[c] 14 ±0.35	[e]10.75 ±0.48	[e]15.75 ±0.71	[c]12 ±0.39	[e]8.75 ±0.73	[e]91.25 ±2.01

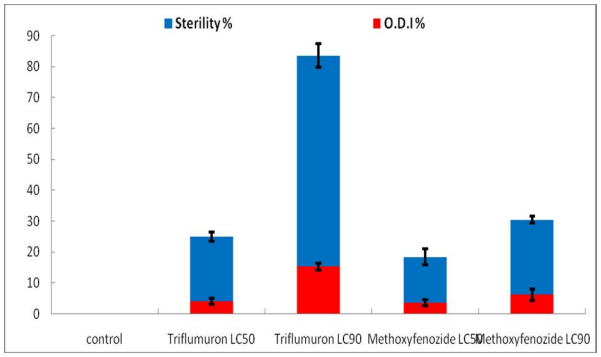

Fig. (4) Effect of Triflumuron and Methoxyfenozide on O.D.I & Sterility of *S. littoralis* treated as 2nd larval instar. Graph shows percent of (4 biological replicates) means ± SE (Student's t-test, p= 0.001).

Fig. (5) morphogenic abnormalities in treated 2nd larval instar, (A) Normal 2nd larval instar (dorsal, lateral and abdominal view), (B) Shrinked 2nd larval instar with greenish chitinization on thorax, (C) 3rd larval instar failed to shed the exuvia of 2nd instar and (D) Intermediate instar between 2nd and 3rd instars showed slight chitinization.

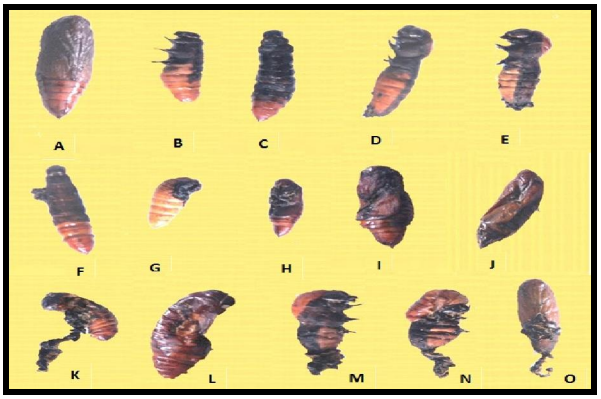

Fig. (6) morphogenic abnormalities in pupal stage, (A) Normal pupa (B-F) Different shapes of larval-pupal intermediates (G) Small discolored soft-shinned pupa with the rest of larval cuticle around the thorax and head (H) Shrinked pupa produced dwarf adult (I) pupae with large malformed head and small abdomen (J) Giant pupa with very large malformed head and very small head (K) Pupa failed in shedding off the larval exuvium (L) C- shaped pupa (M-O) Pupa cannot escape from their larval exuvium.

Catalytic domains of GH family 18 chitinases and endoglycosidases comprises active site consensus motif [D-G-V-D-I-D-W-E] residues (Frederiksen ***et al.,***2013), in the present study this motif was found to be located in the translated protein of normal larvae at positions 105 to 112 amino acids, Triflumuron, interfered with this active site motif by replacing (W) at position 111 with (F) and by replacing (E) at position 112 with (D) and on the other hand Methoxyfenozide replacing (W) at position 111 with (F), The glutamate E112 was most

important for catalytic activity; its substitution by glutamine or even by the negatively charged aspartate led to a complete loss of enzymatic activity **(Merzendorfer; 2013)**, tryptophan W111 within motif appears to be necessary for optimal enzyme activity but is not required for chitin binding **(Huang *et al.*,2000)**, in addition to the other detected mutations which resulted in amino acids at positions [65-156-201-244-317] for Triflumuron and for Methoxyfenozide at positions [156-234].

In conclusion, the results presented in this context, not only highlights the mode of action of IGRs during the process of chitin formation and degradation in cotton leafworm (*Spodoptera littoralis*) larvae as a target and model insect, it also gives another explanation for the bioassays obtained results and malformations, and demonstrate the feasibility to developing a bioassay to screen this target gene for the construction transgenic plants and bio-insecticides for pest control.

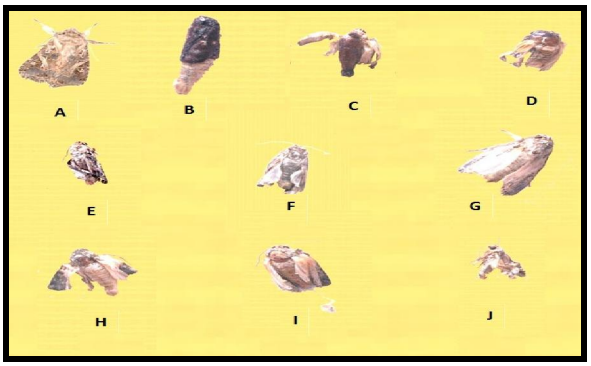

Fig. (7) morphogenic abnormalities in adult stage, (A) Normal adult (B) Pupal-adult intermediate with head and thorax enclosed by old cuticle of pupa (C) Pupal – adult intermediate with head, thoracic legs and wings are free (D) Adult with twisted wings (E) Adult with poorly developed crumpled twisted wings (F) Frizzled wings, absence of legs and malformed abdomen (G) Moth with poorly developed crumbled wings (H) Adult with malformed abdominal tip, poorly developed curled wings and absence of right antenna (I) Adult with twisted wings (J) Small adult with twisted wings.

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Normal          1 GUGAAGGAGAUGGAGGGCAGCUUCCAGGCCCUGCAGAGGAGCUGCAGCGGCAGGGAGGACUUCAAGGUGAGCAUCCACGACCCCUGGGCCGCCCUGCAGAAGCCCCAGAAGGGCCUGAGCAGCUGGAACGAGCCC 135
Triflumuron     1 GUGAAGGAGAUGGAGGGCAGCUUCCAGGCCCUGCAGAGGAGCUGCAGCGGCAGGGAGGACUUCAAGGUGAGCAUCCACGACCCCUGGGCCGCCCUGCAGAAGCCCCAGAAGGGCCUGAGCAGCUGGAACGAGCCC 135
Methoxyfenozide 1 GUGAAGGAGAUGGAGGGCAGCUUCCAGGCCCUGCAGAGGAGCUGCAGCGGCAGGGAGGACUUCAAGGUGAGCAUCCACGACCCCUGGGCCGCCCUGCAGAAGCCCCAGAAGGGCCUGAGCAGCUGGAACGAGCCC 135

cons            1 ************************************************************************************************************************************ 135


Normal        136 UACAAGGGCAACUUCGGCCAGCUGAUGAUGCUGAAGCAGGCCAGGCCCGACCUGAAGAUCCUGCCCAGCGUGGGCGGCUGGACCCUGGCCGACCCCUUCUUCUUCUUCACCGACGAGGUGAAGAGGCACAGGUUC 270
Triflumuron   136 UACAAGGGCAACUUCGGCCAGUGAUGAUGCUGAAGCAGGCCAGGCCCGACCUGAAGACCCUGCCCAGCGUGGGCGGCUGGACCCUGGCCGACCCCUUCUUCUUCUUCACCGACGAGGUGAAGAGGCACAGGUUC 270
Methoxyfenozide 136 UACAAGGGCAACUUCGGCCAGCUGAUGAUGCUGAAGCAGGCCAGGCCCGACCUGAAGAUCCUGCCCAGCGUGGGCGGCUGGACCCUGGCCGACCCCUUCUUCUUCUUCACCGACGAGGUGAAGAGGCACAGGUUC 270

cons          136 ************************************************************ ************************************************************************ 270


Normal        271 GUGGCCAGCGUGAAGGACUUCCUGGAGACCUGGAAGUUCUUCGACGGCGUGGACAUCGACUGGGAGUUCCCCGGCGGCAAGGGCGGCCAACCCCGACCUGGGCGCCCCCGAGGACGGCCACAUCUACGUGCAGCUG 405
Triflumuron   271 GUGGCCAGCGUGAAGGACUUCCUGGAGACCUGGAAGUUCUUCGACGGCGUGGACAUCGACUUCGACUUCCCCGGCGGCAAGGGCGGCCAACCCCGACCUGGGCGCCCCCGAGGACGGCCACAUCUACGUGCAGCUG 405
Methoxyfenozide 271 GUGGCCAGCGUGAAGGACUUCCUGGAGACCUGGAAGUUCUUCGACGGCGUGGACAUCGACUUCGAGUUCCCCGGCGGCAAGGGCGGCCAACCCCGACCUGGGCGCCCCCGAGGACGGCCACAUCUACGUGCAGCUG 405

cons          271 ************************************************************  ** ************************************************************************ 405


Normal        406 AUGAAGGAGCUGAGGGAGAUGCUGAACGAGCUGAGCGCCAAGACCGGCAAGAAGUACGAGCUGACCAGCGCCAUCAGCAGCGGCUGGGACAAGAUCCAGGUGGUGGACUACAAGGAGGCCCAGCAGUACAUGGAC 540
Triflumuron   406 AUGAAGGAGCUGAGGGAGAUGCUGAACGAGCUGAGCGCCAAGACCGCCAAGAAGUACGAGUUCACCAGCGCCAUCAGCAGCGGCUGGGACAAGAUCCAGGUGGUGGACUACAAGGAGGCCCAGCAGUACAUGGAC 540
Methoxyfenozide 406 AUGAAGGAGCUGAGGGAGAUGCUGAACGAGCUGAGCGCCAAGACCGGCAAGAAGUACGAGUUCACCAGCGCCAUCAGCAGCGGCUGGGACAAGAUCCAGGUGGUGGACUACAAGGAGGCCCAGCAGUACAUGGAC 540

cons          406 ************************************************************ * ************************************************************************ 540


Normal        541 CACAUCUUCCUGAUGAGCUACGACUUCAAGGGCGCCUGGAGCAACGACACCCUGGGCCACCAGGCCGGCCUGUACGCCCCCGCCUGGAACCCCAAGGAGACCUACACCACCGACUUCGGCGUGAAGUUCCUGCUG 675
Triflumuron   541 CACAUCUUCCUGAUGAGCUACGACUUCAAGGGCGCCUGGAGCAACGACACCCUGGGCCACCUGGCCGGCCUGUACGCCCCCGCCUGGAACCCCAAGGAGACCUACACCACCGACUUCGGCGUGAAGUUCCUGCUG 675
Methoxyfenozide 541 CACAUCUUCCUGAUGAGCUACGACUUCAAGGGCGCCUGGAGCAACGACACCCUGGGCCACCAGGCCGGCCUGUACGCCCCCGCCUGGAACCCCAAGGAGACCUACACCACCGACUUCGGCGUGAAGUUCCUGCUG 675

cons          541 ************************************************************ ************************************************************************ 675


Normal        676 GCCCAGGGCGUGAGCCCCAAGAAGAUCGUGGUGGGCGUGGCCAUGUACGGCAGGGGCUGGACCGGCGUGAACGGCUACAAGGACGGCAACCCCUUCACCGGCGUGGCCACCGGCCCCGUGAAGGGCACCUGGCAG 810
Triflumuron   676 GCCCAGGGCGUGAGCCCCAAGAAGAUCGUGGUGGGCGUGGCCAUGUACGGCAGGAGGUGGACCGGCGUGAACGGCUACAAGGACGGCAACCCCUUCACCGGCGUGGCCACCGGCCCCGUGAAGGGCACCUGGCAG 810
Methoxyfenozide 676 GCCCAGGGCGUGAGCCCCAAGAAGAGCGUGGUGGGCGUGGCCAUGUACGGCAGGGGCUGGACCGGCGUGAACGGCUACAAGGACGGCAACCCCUUCACCGGCGUGGCCACCGGCCCCGUGAAGGGCACCUGGCAG 810

cons          676 ************************* ************************ * ************************************************************************ 810


Normal        811 GACGGCGUGGUGGACUACAGGGAGAUCGCCAACGAGAUCGCCCAGGGCAAGUGGGAGUACCACUACGACAAGGUGGCCCAGGCCCCCUACGUGUUCAGGAAGGAGACCGGCGACCUGAUCACCUACGACGACGCC 945
Triflumuron   811 GACGGCGUGGUGGACUACAGGGAGAUCGCCAACGAGAUCGCCCAGGGCAAGUGGGAGUACCACUACGACAAGGUGGCCCAGGCCCCCUACGUGUUCAGGAAGGAGACCGGCGACCUGAUCACCUACGACGACGCC 945
Methoxyfenozide 811 GACGGCGUGGUGGACUACAGGGAGAUCGCCAACGAGAUCGCCCAGGGCAAGUGGGAGUACCACUACGACAAGGUGGCCCAGGCCCCCUACGUGUUCAGGAAGGAGACCGGCGACCUGAUCACCUACGACGACGCC 945

cons          811 ************************************************************************************************************************************ 945


Normal        946 AGGAGCACCAUCGAGAAGGCCAAGUACGUGAGGAACAACAAGCUGGGCGGCCUGUUCGCCUGGGAGGUGGACGCCGACAACGGCGACAUCCUGAACGCCAUGAACAUGGGCCUGGGCAACAACGCC 1071
Triflumuron   946 AGGUGGACCAUCGAGAAGGCCAAGUACGUGAGGAACAACAAGCUGGGCGGCCUGUUCGCCUGGGAGGUGGACGCCGACAACGGCGACAUCCUGAACGCCAUGAACAUGGGCCUGGGCAACAACGCC 1071
Methoxyfenozide 946 AGGAGCACCAUCGAGAAGGCCAAGUACGUGAGGAACAACAAGCUGGGCGGCCUGUUCGCCUGGGAGGUGGACGCCGACAACGGCGACAUCCUGAACGCCAUGAACAUGGGCCUGGGCAACAACGCC 1071

cons          946 *** * ************************************************************************************************************************ 1071
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Fig. (8) Alignment of gene sequences.

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Normal              1 VKEIEGSFQALQRSCSGREDFKVSIHDPWAALQKPQKGLSSWNEPYKGNFGQLMMLKQARPDL   63
Triflumuron         1 VKEIEGSFQALQRSCSGREDFKVSIHDPWAALQKPQKGLSSWNEPYKGNFGQLMMLKQARPDL   63
Methoxyfenozide     1 VKEIEGSFQALQRSCSGREDFKVSIHDPWAALQKPQKGLSSWNEPYKGNFGQLMMLKQARPDL   63

cons                1 ************************************************************   63


Normal             64 KILPSVGGWTLADPFFFFTDEVKRHRFVASVKDFLETWKFFDGVDIDWEFPGGKGANPDLGAP   126
Triflumuron        64 KTLPSVGGWTLADPFFFFTDEVKRHRFVASVKDFLETWKFFDGVDIDFDFPGGKGANPDLGAP   126
Methoxyfenozide    64 KILPSVGGWTLADPFFFFTDEVKRHRFVASVKDFLETWKFFDGVDIDFEFPGGKGANPDLGAP   126

cons               64 * **********************************************:**************   126


Normal            127 EDGHIYVQLMKELREMLNELSAKTGKKYELTSAISSGWDKIQVVDYKEAQQYMDHIFLMSYDF   189
Triflumuron       127 EDGHIYVQLMKELREMLNELSAKTGKKYEFTSAISSGWDKIQVVDYKEAQQYMDHIFLMSYDF   189
Methoxyfenozide   127 EDGHIYVQLMKELREMLNELSAKTGKKYEFTSAISSGWDKIQVVDYKEAQQYMDHIFLMSYDF   189

cons              127 ****************************:*********************************   189


Normal            190 KGAWSNDTLGHQAGLYAPAWNPKETYTTDFGVKFLLAQGVSPKKIVVGVAMYGRGWTGVNGYK   252
Triflumuron       190 KGAWSNDTLGHLAGLYAPAWNPKETYTTDFGVKFLLAQGVSPKKIVVGVAMYGRRWTGVNGYK   252
Methoxyfenozide   190 KGAWSNDTLGHQAGLYAPAWNPKETYTTDFGVKFLLAQGVSPKKSVVGVAMYGRGWTGVNGYK   252

cons              190 *********** ******************************** ********* ********   252


Normal            253 DGNPFTGVATGPVKGTWQDGVVDYREIANEIAQGKWEYHYDKVAQAPYVFRKETGDLITYDDA   315
Triflumuron       253 DGNPFTGVATGPVKGTWQDGVVDYREIANEIAQGKWEYHYDKVAQAPYVFRKETGDLITYDDA   315
Methoxyfenozide   253 DGNPFTGVATGPVKGTWQDGVVDYREIANEIAQGKWEYHYDKVAQAPYVFRKETGDLITYDDA   315

cons              253 ************************************************************   315


Normal            316 RSTIEKAKYVRNNKLGGLFAWEVDADNGDILNAMNMGLGNNA  357
Triflumuron       316 RWTIEKAKYVRNNKLGGLFAWEVDADNGDILNAMNMGLGNNA  357
Methoxyfenozide   316 RSTIEKAKYVRNNKLGGLFAWEVDADNGDILNAMNMGLGNNA  357

cons              316 * ***************************************  357
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Fig. (9) Alignment of the encoded amino acids.

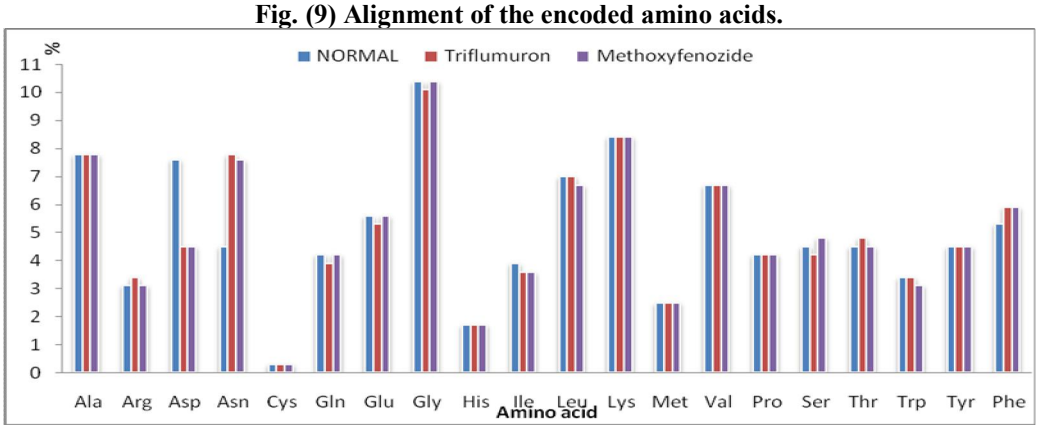

Fig. (10) Histogram of amino acids percentages

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