

## TOXICOLOGICAL, BIOCHEMICAL AND ULTRASTRUCTURAL ALTERATIONS INDUCED BY CHLORFLUAZURON AND *BACILLUS THURINGIENSIS* (DIPLE D.F.) ON *PECTINOPHORA GOSSYPIELLA* (SAUNDERS) LARVAE

Rania, M. El Shennawy

Bollworms Dept., Plant Protection Research Institute, ARC, Dokki, Giza, Egypt.

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### Abstract

Effects of the lethal concentrations of Chlorfluazuron and *Bacillus thuringiensis* (Diple D.f.) against *Pectinophora gossypiella* (Saund.) larvae treated as neonate were studied recently. The obtained results indicated that, Chlorfluazuron treatment showed great toxicity compared to *B. thuringiensis*. The biochemical response of 10-days old larvae was studied by the evaluation of the total soluble protein, total lipids, and transaminases activities: Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), and the carbohydrate hydrolyzing enzymes (trehalase, invertase and amylase). The total protein content decreased to 43.26mg/dl and 26.24mg/dl for chlorfluazuron and *Bacillus thuringiensis* treatments, respectively compared to untreated control (100.1 mg/dl). In contrast, the total lipid levels were increased for the same compounds, recording values: 132.73 and 87.87 mg/dl, respectively, compared to control (36.51 mg/dl). Moreover, a high increase in the activity of all Carbohydrate hydrolyzing enzymes induced by both treatments compared to control. The activity of AST enzyme was declined to 5.44 and 12.66 mg glucose released/gm body weight/min for Chlorfluazuron and *B. thuringiensis*, respectively, compared to control (14.62 mg glucose released/gm body weight/min). In the contrary, a great increase in the ALT activity was recorded with values: 129.49 and 159.61 mg/dl for the same compounds, respectively compared to untreated check (85.36 mg/dl). Newly hatched larvae of *P. gossypiella* treated with Chlorfluazuron as one of chitin biosynthesis inhibitors showed some histopathological alternations of the cuticle layers. The exo-cuticle became thicker with variable staining affinity, while the endo-cuticle became thin and faintly stains. The epidermal layer was formed mostly with irregularly distributed cubical cells varied in height and staining affinity. Furthermore, histopathological alternations, of midgut was obtained for *Bt* treated larvae of *P. gossypiella* representing extensive damage of the epithelial columnar cells with increased cytoplasm vacuolation at the *Bt* toxins. In addition, ultrastructure studies conjugated with observations for both Chlorfluazuron and *Bt* treated and untreated PBW larvae were massive supported by transmission electron microscopy (TEM).

**Keywords:** pink bollworm; *Pectinophora gossypiella*, (PBW); chlorfluazuron, *Bacillus thuringiensis*; toxicity; *Bt*; AST; ALT; histopathological alternations, electron microscopy.

## INTRODUCTION

The pink bollworm, *Pectinophora gossypiella* (Saunders) is one of the injurious insect pest present a major threat to economical production of cotton, *Gossypium spp* in many cotton-growing regions of the world. The present work aimed to investigate the effects of two control agents on *P. gossypiella* larvae.

Chlorfluazuron compound is one of insect growth regulator that prevent chitin synthesis and provides effective control of various pest insects, especially Lepidoptera, in corn, cotton, fruit trees and vegetables at low dose rates.

*Bacillus thuringiensis* is a natural bacterium that has been used since 1950s as an instrument of biological control for different agricultural fields and vectors of animal and human diseases (Lambert and Peferoen, 1992). It is considered the most common environmental-friendly alternate to chemical insecticides which are used for control. Different strains of *B. thuringiensis* produce crystalline spores, contain one or more toxic proteins known as  $\delta$ -endotoxins that are highly specific against different species of lepidopteran, coleopteran and dipteran insects (Denolf *et al.*, 1993 and, Estada and Ferre 1994).

When toxic chemical pesticides are used in the management of injurious insect pests, the various biochemical factors of the pests are modified leading to the death of the organisms and changes in the organisms preventing its reproductive capabilities (Nath and Kumar, 1999).

Generally, amino transferases (transaminases), key enzymes in linking cellular metabolism of proteins and carbohydrates were drastically affected by pesticide toxicity. Various biochemical reactions carried out in insects by a group of amino-transferases enzymes as a result of the balanced amino acid pool. Study of hemolymph proteins is essential to understand the impact of pesticides on lepidopteran larvae (Mordue and Goldworthy, 1973 and Meisner *et al.* 1986).

## MATERIALS AND METHODS

### 1. Tested insect:

The laboratory strain, of pink bollworm, *P. gossypiella*, neonate larvae used in this study was obtained from the laboratory colony of Bollworms Research Department, Plant

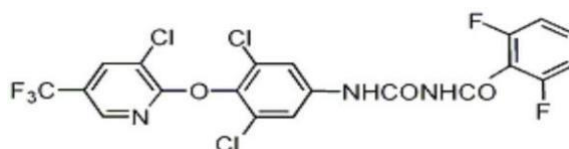
Protection Research Institute; Agricultural Research Center (ARC) and reared for several generations away from any contamination with insecticides.

The pink bollworm larvae were reared on an artificial modified diet as illustrated by Rashad and Ammar (1985).

## 2. Tested Compounds:

### 2.1- Common name: Chlorfluazuron

**Trade name:** Atabron 5% EC. (10 -100 g a.i/ha).



### 2.2- Biocide:

**Common name:** *Bacillus thuringiensis* ssp. *Kurstaki*

**Trade name:** DiPel.<sup>®</sup>. D.f. (45%).

**Composition:** *Bacillus thuringiensis* is an aerobic spore-forming Gram-positive. Crystals of protein (the delta-endotoxin) are also formed.

## 3. Toxicological studies:

Toxicity of both Chlorfluazuron and *Bacillus thuringiensis* compounds against newly hatched larvae of *P. gossypiella* was calculated. Series of aqueous concentrations for each compound were prepared as follow:

(a) Seven concentrations ranged from 25 to 0.3906 ppm of Chlorfluazuron.

(b) Four concentrations ranging from 128000 to 16000.0 I. U of *Bt.*

These concentrations were tested against newly hatched larvae of PBW. One ml of each concentration was mixed with 30 g of the prepared diet (with no antimicrobial components for *Bt.* treatment). Each treated diet was folded into 3 Petri dishes (9 cm in diameter). Twenty healthy newly hatched larvae of PBW were gently transferred to the diet surface by using a soft hair brush. Another group of 3 dishes with normal diet was mixed with the same volume of distilled water and considered as control. The larvae were allowed to feed on the diets for 1 hr at 26±1°C and 75±5% relative humidity. The

alive larvae were transferred singly into glass tubes (2 x 7 cm) containing 2 g of untreated artificial diet. The tubes were plugged with pieces of cotton wool and incubated at the above conditions. The percentages of larval mortalities were recorded after 48 hr for DiPel.®. D.f. and at the 6<sup>th</sup> day for Atabron treatments, and corrected according to Abbott's formula (1925). Values of LC<sub>50</sub> and LC<sub>90</sub> were calculated according to Finny (1971).

#### 4. Biochemical studies:

Biochemical quantities were done at Physiology Dept., Plant Protection Research Institute.

##### 4.1. Preparation of the samples.

Pink bollworm larvae of ten days old treated as neonate with LC<sub>50s</sub> were used for biochemical quantification. In distilled water. Samples were homogenized using a Teflon homogenizer, then centrifuged at 5000 r.p.m. for 10 minutes at 5°C. A same sample of untreated larvae were used as control check.

##### 4. 2. Determination of total soluble protein.

Colorimetric evaluation of total soluble protein in total body homogenate larvae was estimated, as described by Gornall *et al.* (1949).

##### 4. 3. Determination of total lipids.

The total lipids were valuated according to the techniqueof (Schmit, 1964).

##### 4. 4. Determination of enzymes activities:

#### 4. 4.1. Transaminase enzymes.

Alanine transaminase (ALT) and Aspartate transaminase (AST) enzyme activities were evaluated colorimetrically according to method of Reitman and Frankle. (1957).

##### 4.4.2. Carbohydrate hydrolyzing enzymes.

Activities of trehalase, amylase and invertase enzymes, were quantified according to the method of Ishaaya and Swiriski, (1976).

#### 5. Histological and ultrastructure examinations:

These experiments were done at Electron Microscope Unit of Assiut University. Specimens of PBW 10 day's old larvae of (for both treated and untreated check) were killed by chloroform, and fixed in aqueous neutral buffer. A block of 1 x 2 mm was taken from each sample at the level of the 4<sup>th</sup> abdominal segment, and kept in 5 % cold glutaraldehyde directly after

dissecting for 24 – 48 hr. Samples were then rinsing in cacodylate buffer (pH 7.2) 3 – 4 times for 20 min every time and post fixed in 1 % osmic acid for 2 hr, then washed again 4 times as above. Dehydration by subsequent ascending levels of alcohol (30, 50, 70, 90 and 100% for 2 hr) of each specimen was done. The specimens were implanted epomaraldite mixture as said by (Bozzol and Russell, 1991). From the implanted sectors semi thin sections by L K B extremist - microtome in thickness of 0.5  $\mu$ m were formed to be photographed by using sc30 Olympus camera, ultrathin section in thickness of 500 – 700 A were created using Leica AG ultra-microtome and distinguished in uranyl acetate and lead citrate, as required. The sections were inspected by JEM 100 CXII electron microscope at 80 Kv and photographed by CCD digital camera Model XR- 41.

## RESULTS

- Toxicological studies:**

Toxicity values of the two tested compounds Chlorfluazuron and *B. thuringiensis* against newly hatched larvae of *P. gossypiella* are indicated in Table (1).

Table (1): Toxicity values of Chlorfluazuron and *Bacillus thuringiensis* against newly hatched larvae of *P. gossypiella* under controlled conditions ( $26 \pm 1^\circ\text{C}$  and  $75 \pm 5\% \text{R.H.}$ )

Compound	LC values (ppm)		Slope $\pm$ SE
	LC <sub>50</sub>	LC <sub>90</sub>	
<b>Chlorfluazuron®</b> (Atabron)	17.47	253.360	1.1035 $\pm$ 0.3990
<i>Bacillus thuringiensis</i> (DipleD.f.)®	98430.30	1559100	1.068 $\pm$ 0.287

Based on LC<sub>50</sub> as well as LC<sub>90</sub>, *P. gossypiella* larvae displayed high susceptibility to Chlorfluazuron treatment in comparison to *B. thuringiensis* treatment.

- Biochemical studies:**

The biochemical response of *P. gossypiella* larvae 10-days old treated with the lethal concentrations of each tested compound as well as untreated ones was studied by the evaluation of the total soluble protein, total lipid, transaminases; Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT); activities, and the carbohydrate hydrolyzing enzymes (trehalas, invertase and amylase), respectively.

### 1. Total soluble protein and total lipids:

Table (2) shows the concentrations levels of total soluble protein, total soluble lipid and the changes as a percentage from the control in larval body homogenate. The total protein content decreased to 43.26 and 26.24 mg/dl for chlorfluazuron and *B. thuringiensis* treatments, respectively, compared to the control (100.1 mg/dl).

In contrast, total lipid increased by chlorfluazuron and *B. thuringiensis* treatments to reach 84.97 and 68.59 mg/dl respectively, compared to the control (36.51mg/dl).

**Table (2):** Effect of the tested compounds on total soluble protein and total lipids of *P. gossypiella* treated larvae.

Treatment	Newly hatched larvae			
	Total Soluble Protein		Total Lipids	
	Conc. (mg/dl)	C (%)	Conc. (mg/dl)	C (%)
Chlorfluazuron® (Atabron)	43.26	-56.78	84.97	+132.73
Bacillus thuringiensis (DipleD.f.)®	26.24	-73.79	68.59	+87.87
Control	100.1		36.51	

Change (C) % =  $\frac{\text{Treatment} - \text{Control}}{\text{Control}} \times 100$

The reduction in protein content of Chlorfluazuron treatment might be to match the high energy demand during the state of exhausted carbohydrate. In addition to the overall utilization of protein, the synthesis of protein might be reduced during prolonged toxic stress (Rashad *et al.* (2015) recorded high reduction in total proteins as compared with control when neonate larvae of PBW treated with LC<sub>50</sub> of teflubenzuron compound. In case of *B. thuringiensis* treatments, (Sabry, 2009) were stated noticeable decrease in the total protein at various concentrations in the black cutworm, *A. ipsilon* (Huf.) and 4<sup>th</sup> instar larvae of *S. littoralis*.

On the other hand, the increase in total lipid level might be a protective mechanism of treated individuals to prevent further entry of insecticides (Weismann, 1956). Thus the highest lipid content increase caused by Chlorfluazuron indicated its higher toxicity. These

results are confirmed by those of Kandil *et al.* (2005) found that larvae of PBW *P. gossypiella* treated by diflobenzuron and chlorfluabenzuron caused an increase in total lipid content by one-two times than the control. Moreover, the increase in total lipid caused by chitin synthesis inhibitors is considered to be a reflection of total carbohydrates increase (Raies, 1998).

- Carbohydrate hydrolyzing enzymes:

Carbohydrates are of imperative value since they can be utilized by the insects' body for of energy production or conversion to lipids or proteins. Carbohydrates metabolism is controlled primarily by carbohydrate hydrolyzing enzymes (Al-shannaf *et al.*, 2012). Results represented in Table (3) refer to a great increase in the activity of carbohydrate hydrolyzing enzymes as a result of the above treatments. The increase in the enzymes' activity recorded 227.70 and 219.11 mg glucose released/gm body weight/min for invertase, 103.32 and 94.87 mg glucose released/gm body weight/min for trehalase and 81.12 and 95.17 mg glucose released/gm body weight/min for amylase enzyme induced by chlorfluazuron and *B. thuringiensis* treatments respectively, compared to the control (71.75, 43.71 and 24.36 mg glucose released/gm body weight/min) for the enzymes as described above.

**Table (3):** Effect of the tested compounds on the activity of carbohydrate hydrolyzing enzymes of *P. gossypiella* treated larvae.

Treatment	Newly atched larvae					
	Invertase ( mg/dl)		Trehalase (mg/dl)		Amylase ( mg/dl)	
	SA	RA (%)	SA	RA (%)	SA	RA (%)
Chlorfluazuron (Atabron)®	227.7	217.35	103.32	136.38	81.12	233.01
<i>B. thuringiensis</i> (Diple D.f.)®	219.1	205.38	94.87	117.02	95.17	85.43
Control	71.75		43.71		24.36	

**SA** (Specific activity) as mg glucose /g. b. wt. /min. in case of carbohydrate hydrolyzing enzymes

**RA** (Relative activity %) = Treatment-control x 100 Control

Observations of the present results show great increase in carbohydrate hydrolyzing enzymes in Diple D.f.® treatment followed by Atabron®. The treatments might lead to disturbance in physiological process of PBW treated larvae. These results are in compatible with that obtained by Khedr *et al.* (2002) who recorded an increase in amylase, invertase and trehalase enzymes occurred after treating larvae of by Bioprl® (IGR) treatment.

Kandil *et al.* (2005) recorded an increase in both invertase and trehalase activity after treating newly hatched larvae of *P. gossypiella* with Chlorfluazuron and difluazuron compounds. Also, Sabry and Khedr (2014) indicated significant elevation in the activities of trehalase enzyme in treated the 4<sup>th</sup> instar larvae of *S. littoralis* with the LC<sub>50</sub> levels of tebufenozide, teflubenzuron and methoxyfenozide. Rashad *et al.* (2015) reported high increase in invertase activity in newly hatched larvae of *P. gossypiella* treated with methomyl, pyridalyl and teflubenzuron compounds. On contrary, El-Ghar *et al.* (1995), detected distinct reduction in the carbohydrate hydrolyzing enzymes particularly amylase and invertase after treating 5<sup>th</sup> instar larvae of cotton leaf worm,

*S. littoralis* with sub-lethal concentrations of *B. thuringiensis* (beta-exotoxin of *B. thuringiensis*). Also, Al-shannaf *et al.* (2012) recorded a great reduction in the three carbohydrate hydrolyzing enzymes of *Heliothes. armigera* larvae when treated with both chlorfluazuron and Dipel DF®.

- Transaminase enzymes (ALT and AST):

Data in Table (4) show the activity of transaminase enzymes in 10- days old larvae of *P. gossypiella* treated as neonate with the lethal concentrations of *B. thuringiensis* and chlorfluazuron compounds. Both treatments induced decrease in the activity of AST enzyme with values: 12.66 and 5.44 µg pyruvate /ml for *B. thuringiensis* and chlorfluazuron, respectively, compared to control (14.62 µg pyruvate /ml). In contrast, a noticeable enhancement in the activity of ALT enzyme estimated by 159.61 and 129.49 µg pyruvate /ml for *B. thuringiensis* and chlorfluazuron treatments respectively, compared to control 85.35 µg pyruvate /ml.

**Table (4):** Effect of the tested compounds on the activity of transaminase enzymes of *P. gossypiella* treated larvae.

Treatment	Newly hatched larvae			
	(AST)		(ALT)	
	SA (µg pyruvate /ml)	RA (%)	SA (µg pyruvate /ml)	RA (%)
Chlorfluazuron (Atabron)®	5.44	- 62.79	129.49	51.70
<i>B. thuringiensis</i> (Diple D.f.)®	12.66	-13.41	159.61	86.99
Control	14.62		85.36	

SA (Specific activity) as (µg pyruvate /ml)

RA (Relative activity %) = Treatment-control x 100 Control



The present results (Table 4) conclude that, treated neonate larvae of *P. gossypiella* with LC<sub>50</sub> of both chlorfluazuron and *B.thuringiensis* caused disturbance in the activity of ALT and AST enzymes which may cause disturbance in protein metabolism and synthesis of some particular compounds. Changes in the activity of ALT and AST under the influence of toxicant had been reported by many investigators as Campbell and Ofurum, 1986 and Gill *et al.*, 1990). The present results are in accordance with that of (Ahmed *et al.* 1993), who reported a disturbance in the degrees of AST and ALT of earthworms produced by some insecticides such like diflubenzuron. Abou-Taleb *et al.* (2015), reported significant influences on (ALT) and (AST) enzymes of by treated *S. littoralis* larvae with lufenuron and Chlorfluazuron.

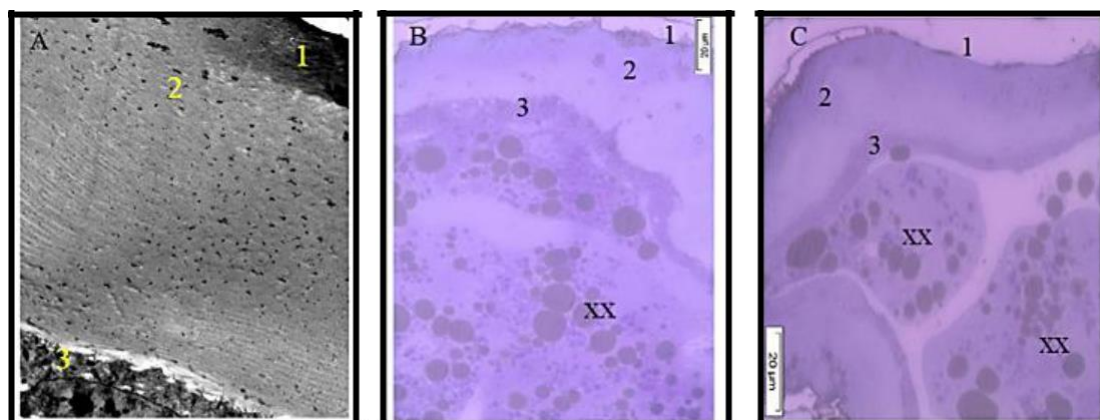
- **Histopathological alternations:**

In this study, histopathological changes occurred as a result of treatments compared to untreated check. It was noted that, the histo-morphological development of the intoxication changed from one treatment to another. Certainly, the cellular destruction as well as the grade of intoxication differ according to the difference of morphological and physiological cells as well as difference of mode of action and the site of action of the tested compounds.

**(a) Effect of Chlorfluazuron treatment on cuticle structure:**

- **Histology investigation:**

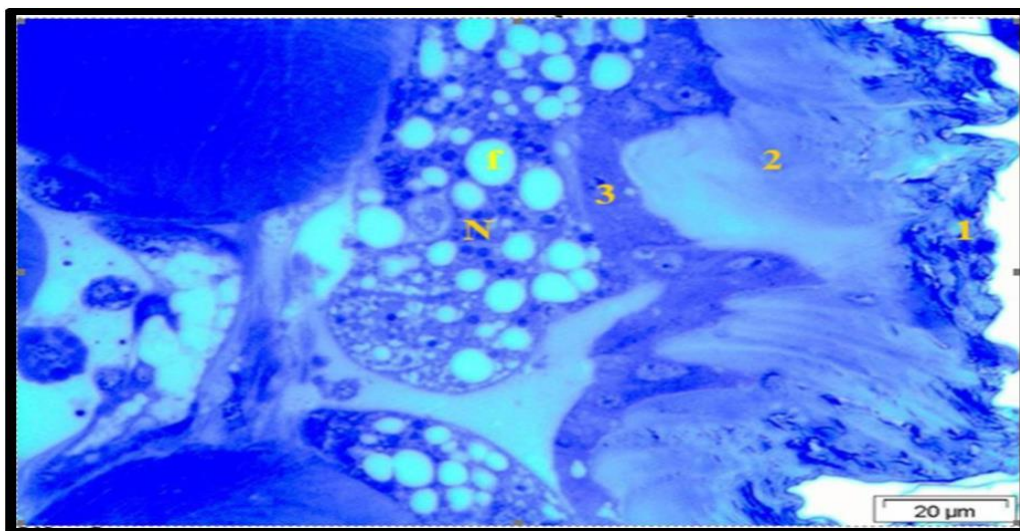
According to light microscopic examination, the cuticle of untreated *P. gossypiella* larva is divided into three layers: (1) the thin non chitinous heavy stained Epicuticle, (2) Procuticle which differentiated into a thin outer tanned and sclerotized exocuticle and a wide inner endocuticle and (3) well-arranged cellular layer of Epidermis, (Fig.1).



**Fig.1: Three light micrograph shots (A, B and C) for the cuticle of untreated *P. gossypiella*. Larva indicated its three layers (1, 2 & 3).**

Notice: Presence of abundant large fat body cells and granulocytes under the epidermal layer or in the body cavity containing large fat globules (xx).

On the other hand, treated newly hatched larvae of *P. gossypiella* with chlorfluazuron as one of chitin biosynthesis inhibitors interlope with chitin formation during molting, causing some histopathological alternation of the cuticle layers (Fig. 2). The exocuticle became thicker with variable staining affinity, while the endocuticle became thin and faintly stain. The epidermal layer was mostly formed with irregularly distributed cuboidal cells varied in height and staining affinity (Fig. 2).

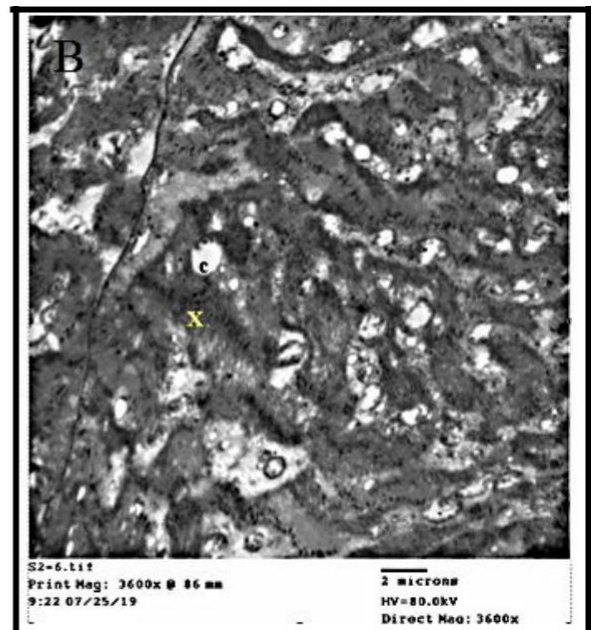
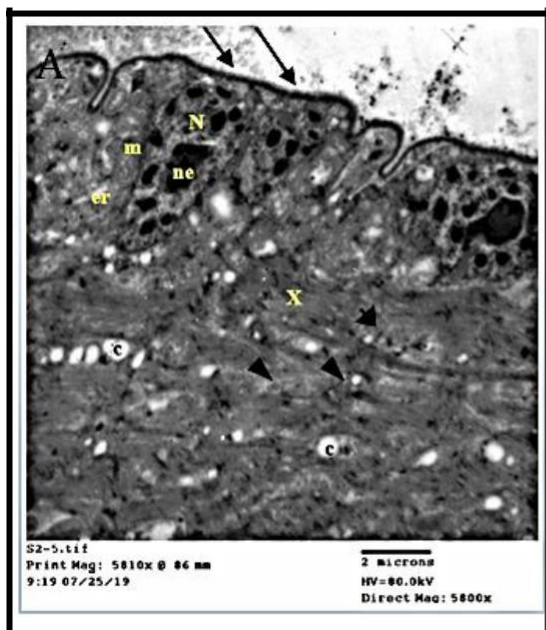


**Fig. 2: Light micrograph of the cuticle of 10 days old *P. gossypiella* larvae treated as neonate with chlorfluazuron indicated the three cuticle layers (1, 2 & 3).**

Notice: the fat body cells having pyknoted nucleus (N), large fat globules (f) and deeply stained granules.

**- Ultrastructure investigation.**

In general, the IGR, Chlorfluazuron interferes with the usual evolution and



disturbs the structure of the different cuticular constituents caused necrotic lesions in cuticle, detachment of cuticle from hypodermis. The epidermal cells were highly impaired being irregular and detached from each other, vacuoles appeared in the cytoplasm and the mitochondria completely lose their normal form and being swollen and fused (Fig. 3-10). The undistinguishable and destroyed epidermal cells may be prevent the pathway of hormonal secretion and thus prevent the coordination between cells, this may induce a great disturbance in the moulting process to produce the new cuticle and shed out of the old one as stated by khaled (2009). Moreover, the present results showed a destruction of the basement membrane and appearance of vacuoles between cuticle and hypodermis. In addition, there was a lack of differentiation between exocuticle and endocuticle thickness of the body. These histopathological changes by Chlorfluazuron agree to great extent with the results recorded by Hassan on *S. littoralis*, (2009) and Ahmed *et al.* (2019) on *Musca domestica*.

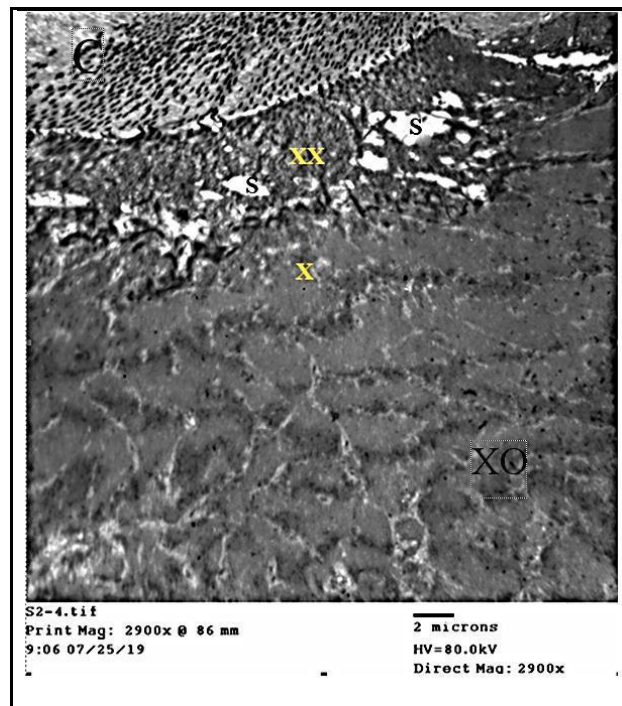
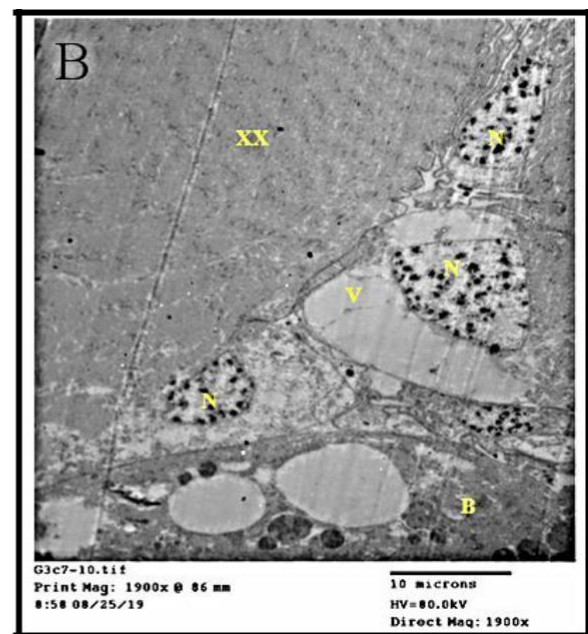
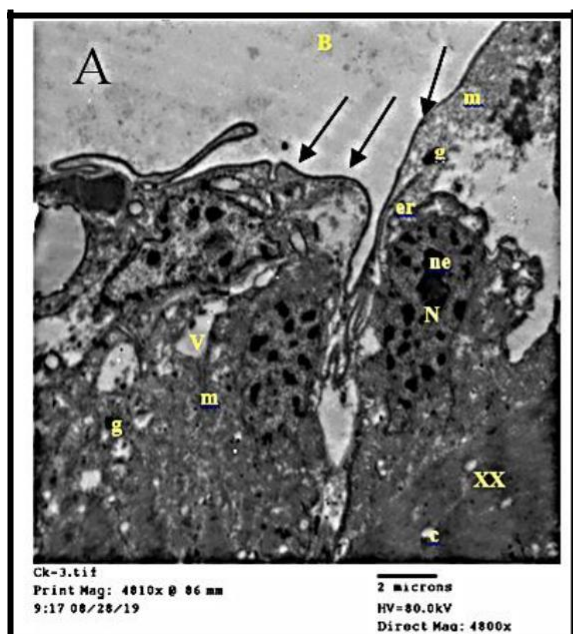


Fig. (3): TEM micrograph of the cuticle of control larva showing (A): one layer of Epidermal cells laying on basement membrane (arrow). The cells contain large nucleus (N) with numerous electron dens chromatin clumps and prominent nucleolus (ne) and the cytoplasm containing cell organelles such as mitochondria (m) and dilated RER (er). (B): Endocuticle of the control larval group showing febrile structure (X) containing numerous pore canals (c). (C): lamellate Exocuticle (XO) contain electron dens fine granules (arrowhead) and the Epicuticle (XX) appeared electron dens contain numerous variable size spaces (s). [T.B. stain].



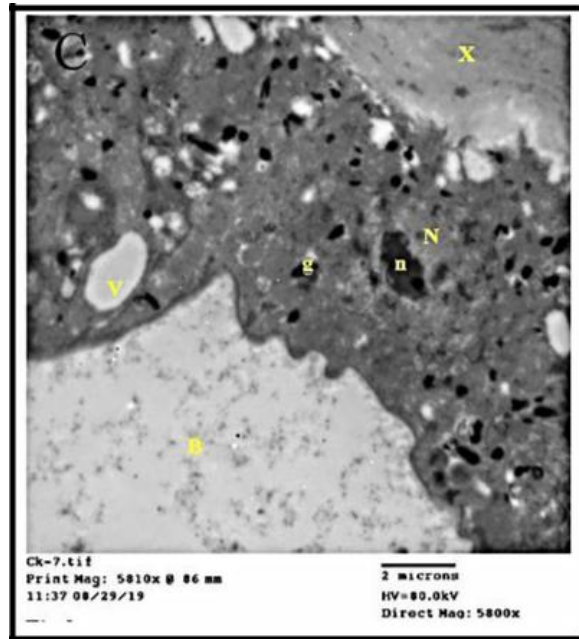
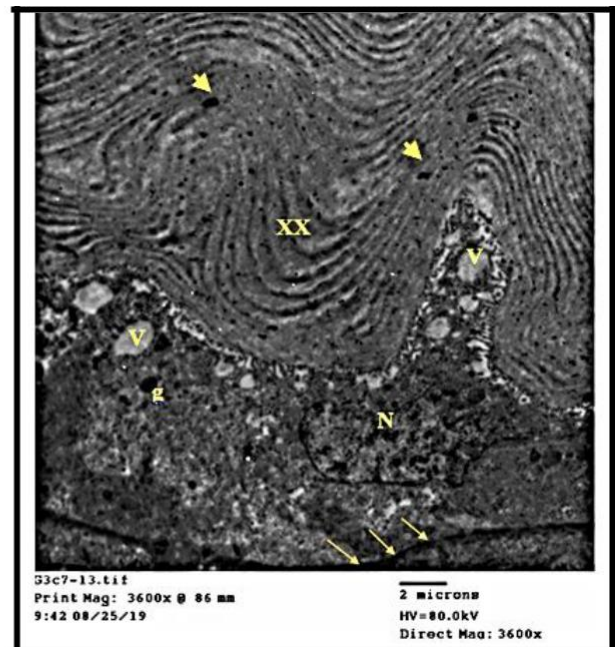
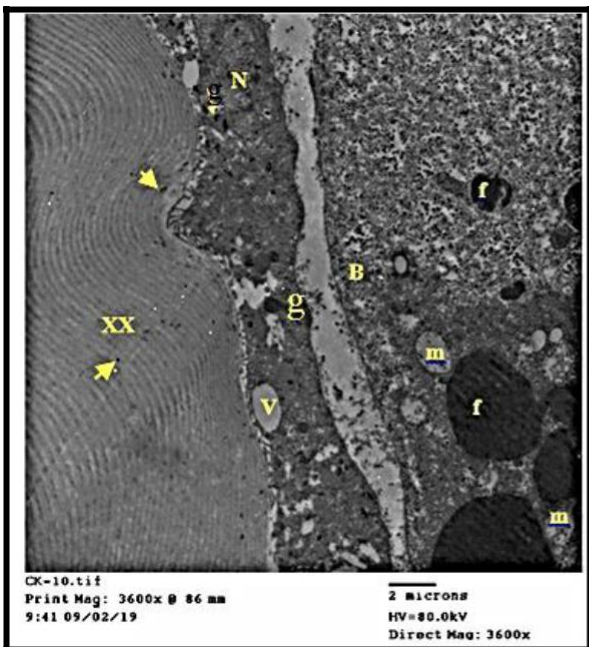


Fig. (4). TEM micrograph of the cuticle of Chlorfluazuron treated larva (A, B and C), showing the epidermal cell layer laying on undulating basement membrane (arrow) toward the body cavity (B). The cell having nucleus (N) contain prominent nucleolus (ne) and cytoplasm contain variable size vacuoles (V), dilated RER (er), electron dens granules (g) and swollen mitochondria (m). The Exodermis (X) appeared in form of light electron dens febrile structure while Endodermis (XX) is lamellate having few pore canal (c) and fin electron dens granules [T.B. stain].



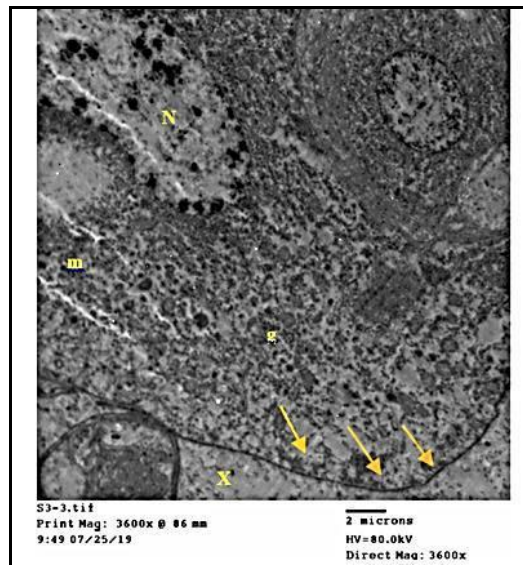


Fig.5. (A&B). TEM. micrograph for the cuticle of Chlorfluazuron treated larva showing marked degenerated epidermal cells laying on basement membrane (arrow), with vacuolated cytoplasm (V) containing electron dens variable size granules (g) and indented nucleus (N) containing numerous electron dens granules. The Endocuticle appeared lamellate in wavy manner (XX) containing electron dens variable size granules (arrowhead) [T.B. stain].

Fig.5. (C). TEM micrograph of the cuticle of Chlorfluazuron treated larva showing the epidermal cells having large vesicular nucleus (N) contain electron dens chromatin and vacuolated cytoplasm contain variable size fin glycogen granules (g), mitochondria (m). Notice presence of fine light electron granules in the body cavity (X). [T.B. stain].

Notice: The body cavity contains abundant amount of glycogen granules (X) and fat globules (f) and vacuoles mitochondria (m) in the degenerated epidermal cell fell in the body cavity.

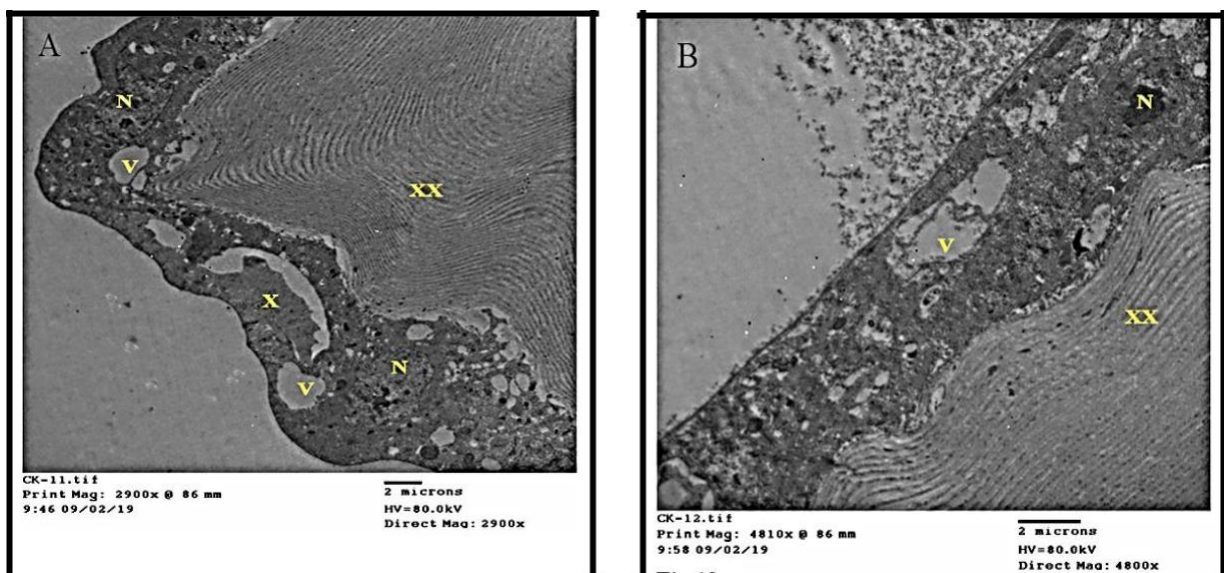


Fig.6. (A &B). TEM micrograph of the cuticle of Chlorfluazuron treated larva showing the epidermal cells having in their cytoplasm variable size vacuoles (V) some of them contain light electron dens material (X) with some condensed granules and their nucleus (N) condensed or pyknosed. The Endocuticle (XX) is lamellated and wavy in appearance. [T.B. stain].

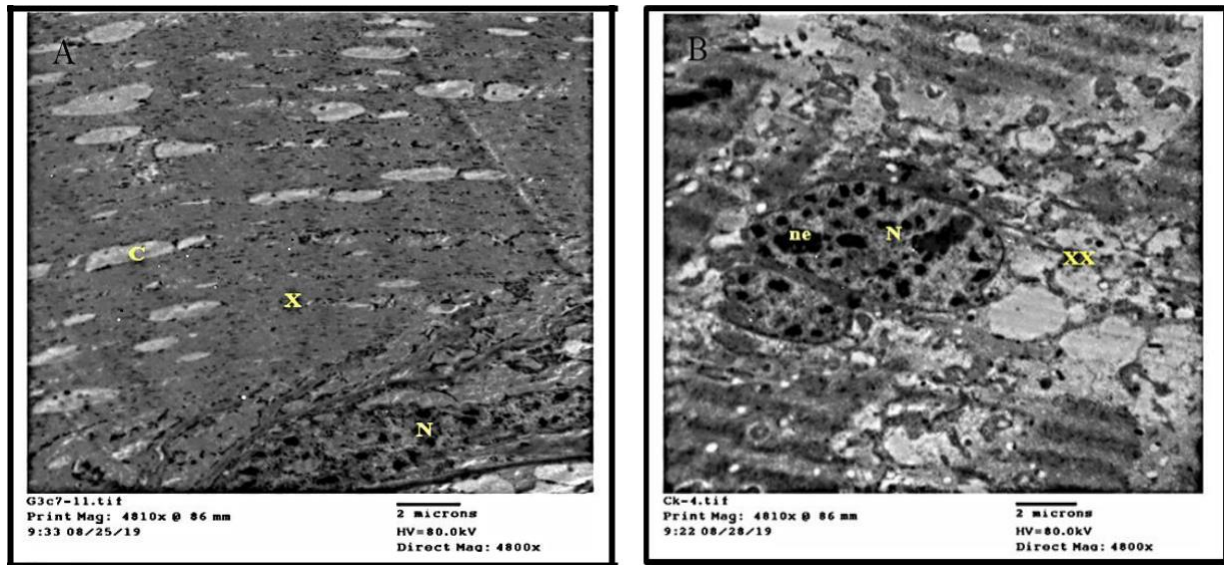


Fig.7. (A&B). TEM micrograph of the cuticle of Chlorfluazuron treated larva showing marked degeneration and lyses of the muscular tissue (XX) and pyknosis or condensation of the nucleus (N) having more than one nucleolus (ne). The Endocuticle (X) formed by fibril structure containing electron dens granules with presence of pore canals (c). [T.B. stain].

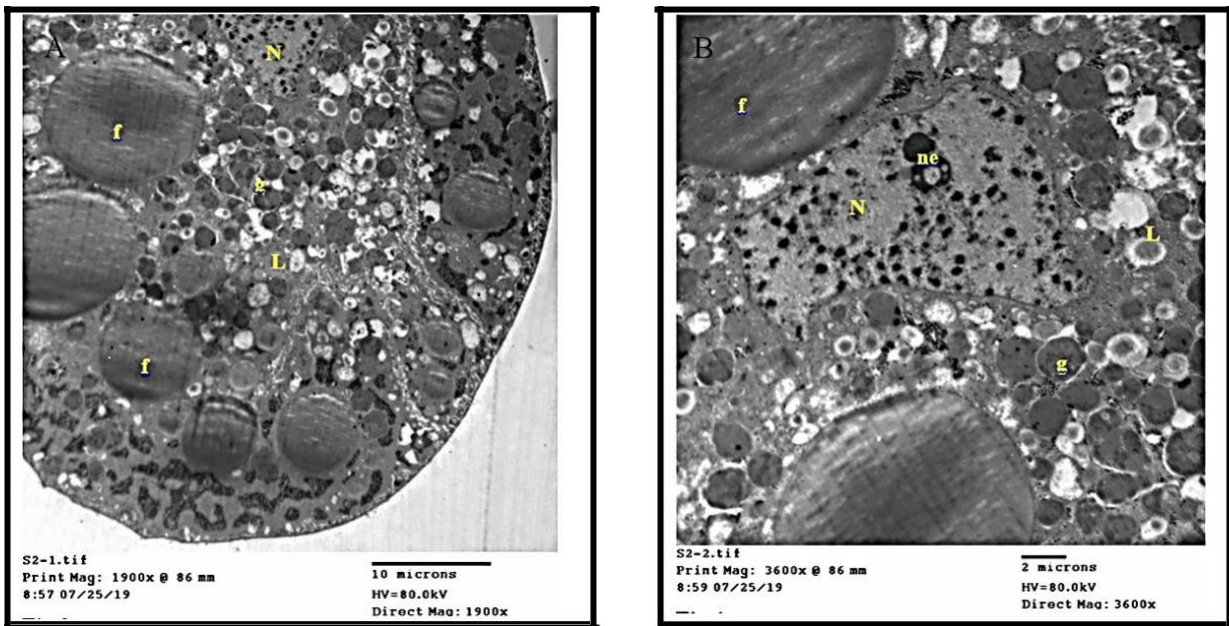


Fig.8 (A&B). TEM micrograph of the granulocytes and fat body cells for untreated larva appeared large cell having large nucleus (N) contain more than one nucleolus (ne) and numerous electron dens chromatin clumps distributed in the nuclear sap. The homogenous cytoplasm contains numerous fat globules (f) varies in their size and two types of granules, electron dens type (g) and light electron dens type (L). [T.B. stain].

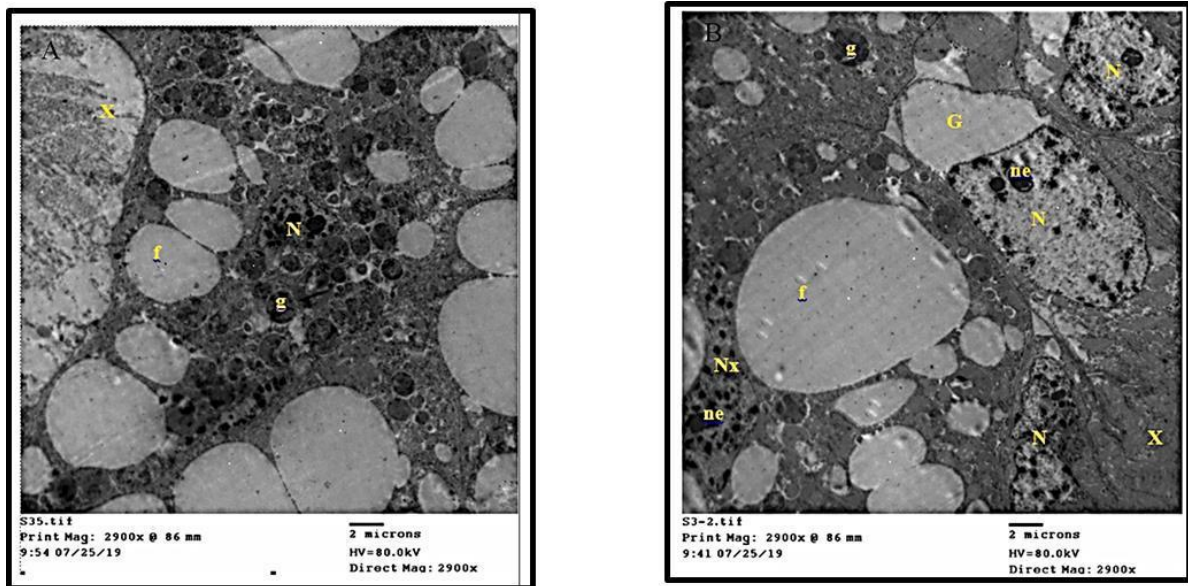


Fig. 9 (A&B)). TEM micrograph of the cuticle of Chlorfluazuron treated larva showing the epidermal cells attached to the fat body cells. The fat body cells contain large fat globules (f), few electron dens granules (g) and compressed nucleus (Nx) contain nucleolus (ne) and electron dens granules in the nuclear sap. The epidermal cells having nucleus (N) contain numerous nucleolus (ne) and electron dens granules in the nuclear sap and presence of variable size vacuoles (V) in the cytoplasm. Notice, presence of parts from the Endocuticle (X) appeared light electron dens. [T.B. stain].

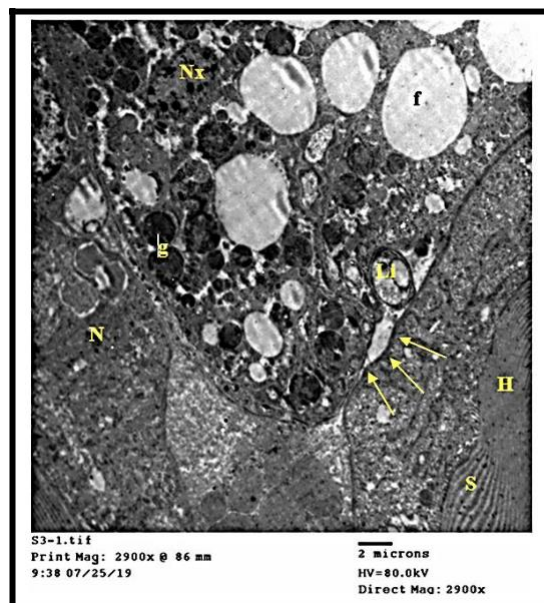


Fig. (10). TEM micrograph of the cuticle of Chlorfluazuron treated larva showing the fat body cells contain numerous fat globules (f), electron dens variable size granules (g), lipofucin granules (Li), and pyknoted or condensed nucleus (Nx) contain numerous electron dens granules in the nuclear sap. The epidermal cells appeared in form of one cell layer laying on basement membrane (arrow) attached to the fat body cells having degenerated cytoplasm and condensed nucleus (N). Notice presence of part from the Endocuticle appeared light electron dens homogenous (H) or lamellate or striated (S). [T.B. stain].



**(b) Effect of *B. thuringiensis* treatment on midgut structure:**

**Histological response:**

The histological structure of the midgut of untreated *P. gossypiella* larvae showed that, it was composed of closely arranged columnar epithelium cells with a rather broad apex, bearing an apical regular microvilli border and every cell containing a large coarsely nucleus (N) occupying a middle position within the cell. Also, small calyx-shaped goblet cells were seen in great numbers between the columnar cells, both types of cells rest on a basement membrane. The epithelium cells shield from the mid gut lumen content by the peritrophic membrane (Fig. 11, A and B).

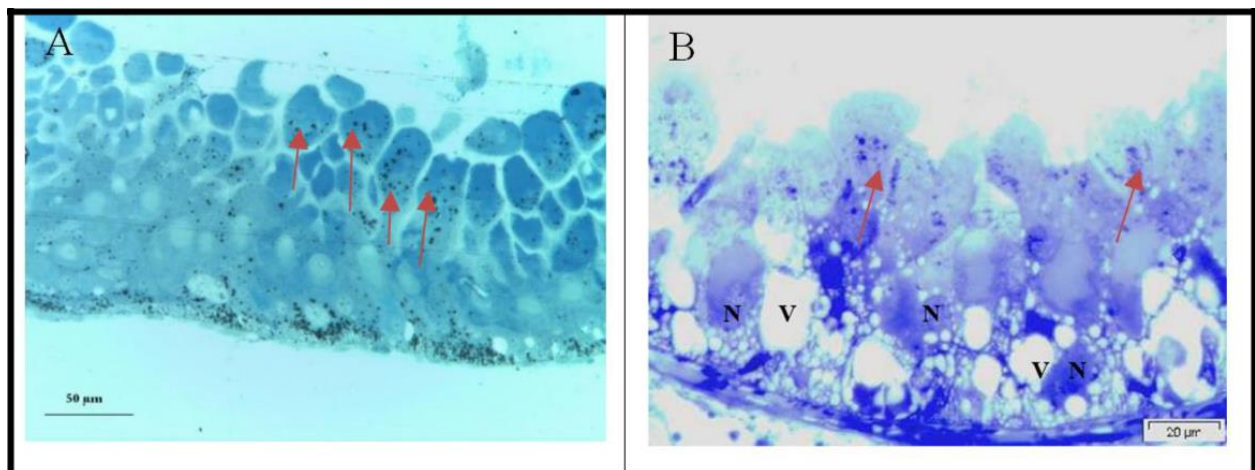


Fig.11 (A&B): Light micrograph of the mid gut of 10 days old *P. gossypiella* untreated larva, indicated the lining epithelium of simple columnar type having vesicular nucleus (N) and its cytoplasm contain numerous variable size vacuoles (v) and few deeply stained granules mostly at the tip of the cells (arrow) .

*B. thuringiensis* crystals ingested by *P. gossypiella* larvae liquefied under the alkaline medium of the gut to release one or more protein toxins that destroy the gut lining. Different histopathological alternation of midgut was obtained for larvae which had been fed a diet containing *B. thuringiensis* resulted in extensive damage. There are a general loosening of the midgut columnar cells from one another and from the basement membrane formed an irregular shapes, high cell vacuolation, and the peritrophic membrane was considerably deteriorated at many regions which caused passing of the

epithelium cell contents into the midgut lumen. In addition, a few pycnotic free nuclei were floating in the lumen and mixed with the peritrophic membrane.(Fig. 12, A and B).

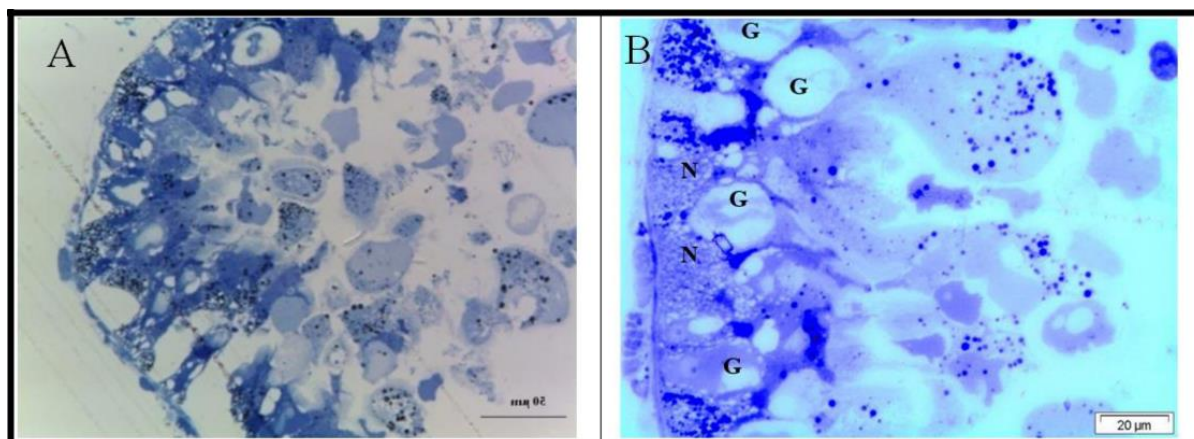


Fig.12 (A&B): Light micrograph of the midgut of 10 days old *P. gossypiella* larva treated as neonate with *B. thuringiensis*, indicated presence of large number of Goblet cells (G) in the mucosa in between the lining epithelium. The lining epithelium of columnar type contain large amount of deeply stained granules in the cytoplasm mostly in the tip of the cell and their nucleus (N) situated at the base of the cell and condensed or pyknotic.

#### - Ultrastructural changes.

Ultrastructure of midgut cell for *P. gossypiella* larvae fed on non-*Bt* treated diet was well organized with thick cytoplasm and intact plasma membrane. On the other hand, feeding newly hatched larvae of *P. gossypiella* on *Bt* contaminated diet affecting their midgut epithelial cells with increasing cytoplasm vacuolation and nuclear chromatin condensation following their ingestion due to *Bt* toxins (Alves and Melo, 2010). *Bt* crystalline inclusions converted to active toxins by trypsin like proteases in the highly alkaline midgut lumen. The activated toxins traverse the peritrophic membrane, bind to the brush border apical membrane of midgut columnar cells at specific receptors, and interleave into the membrane. Pore formation disturbs the ionic inclines and osmotic balance across the apical membrane and ultimately causes the epithelial mid gut cells to lyse (Sousa, 2010). This led to a broad disruption of the epithelium allowing the passage of cellular splinters with cytoplasmic and nuclear matter into the midgut lumen (Castro *et al.*, 2019) which ultimately directed to the mortality of the larvae by feed less or septicemia.

All morphological as well as histological deformation of midgut cells as a result of *Bt* treatment compared to untreated check are illustrated in Fig (13 - 18).

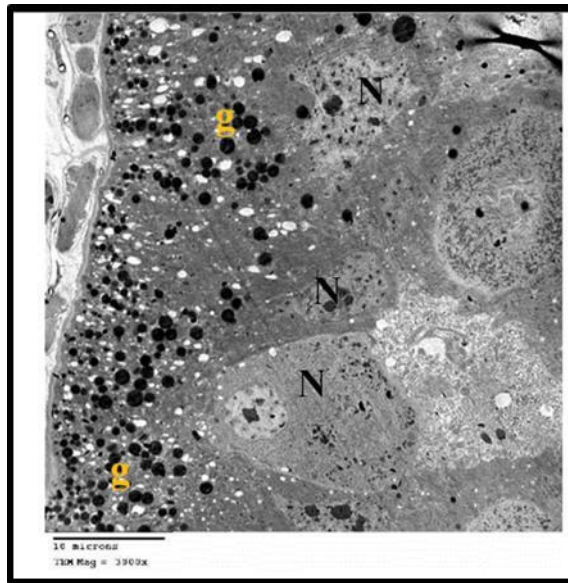


Fig.(13): TEM micrograph of the untreated larval mid gut showing the lining epithelium of columnar type situated on basement membrane having large nucleus (N) contain one or more nucleolus and the cytoplasm contain numerous electron dens variable size granules (g). The lower portion of the cells having large amount of variable size membranous. [T.B. stain].

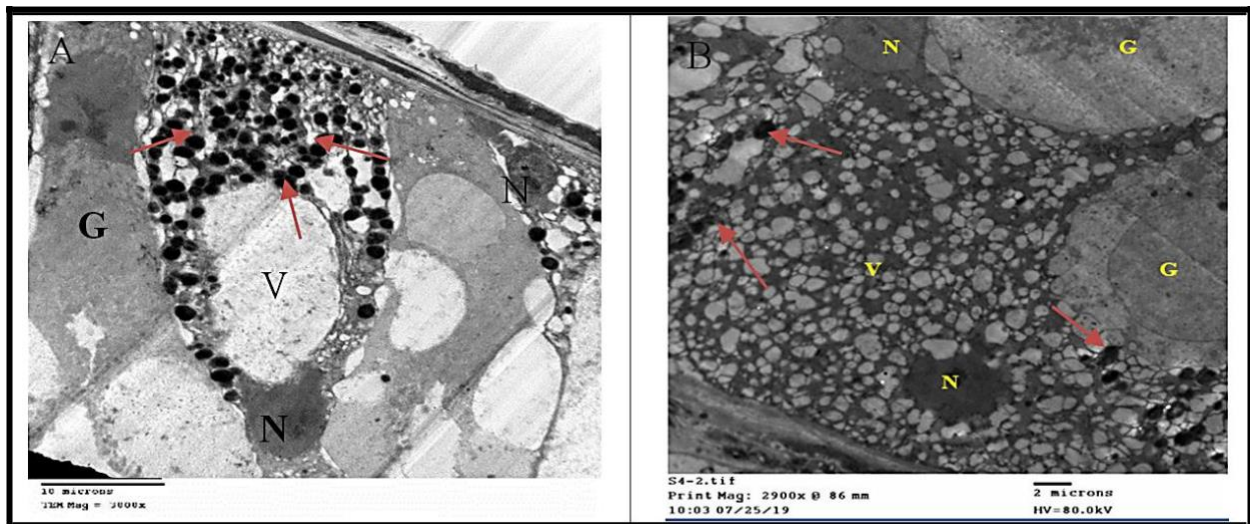


Fig.14 (A&B): TEM micrograph of the midgut of *Bt* treated larva showing presence of numerous Goblet cells (G) with variation in electron density of the mucus content. Condensation of nuclear chromatin of the nucleus (N) of the epithelial lining and presence of large amount of membranous vacuoles (V) in the cytoplasm specially at the lower portion of the cells. Notice presence of numerous electron dens granules (arrow). [T.B. stain].

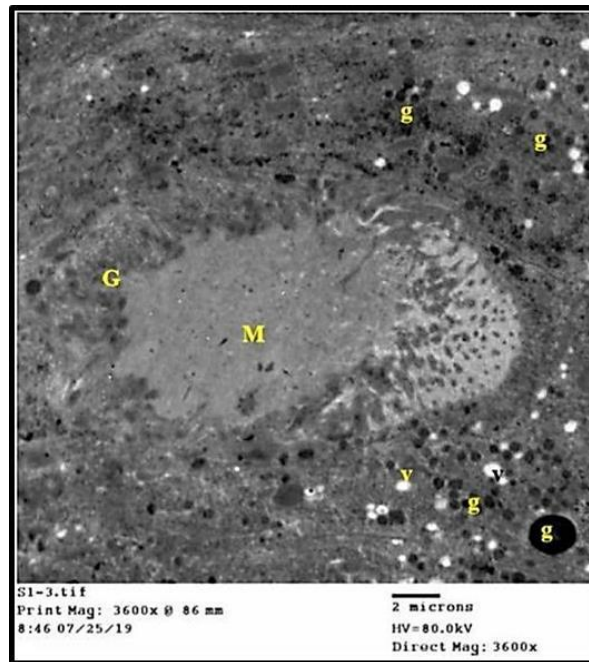


Fig. 15: TEM micrograph of untreated larval midgut showing goblet cell (G) imbedded in the lining epithelium contain light electron dens material (M). The surrounding epithelial cells contain numerous variable size electron dens granules (g) and small vesicles (v). [T.B. stain].

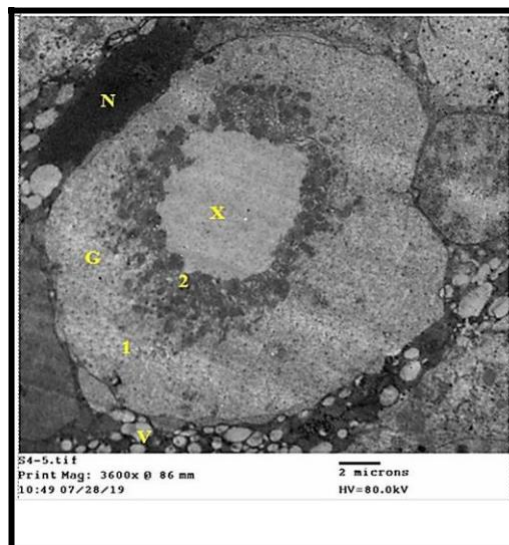


Fig. 16: TEM micrograph of *Bt* treated larval midgut mucosa showing the goblet cell contain two type of mucus fine granular and light electron dens at the periphery (1) and large variable size and electron dens centrally (2) surrounding light electron dens homogenous material (X). The cytoplasm of the goblet cell contains numerous membranous vacuoles (V). Notice the surrounding epithelial cell contain electron dens elongated nucleus (N). [T.B. stain].

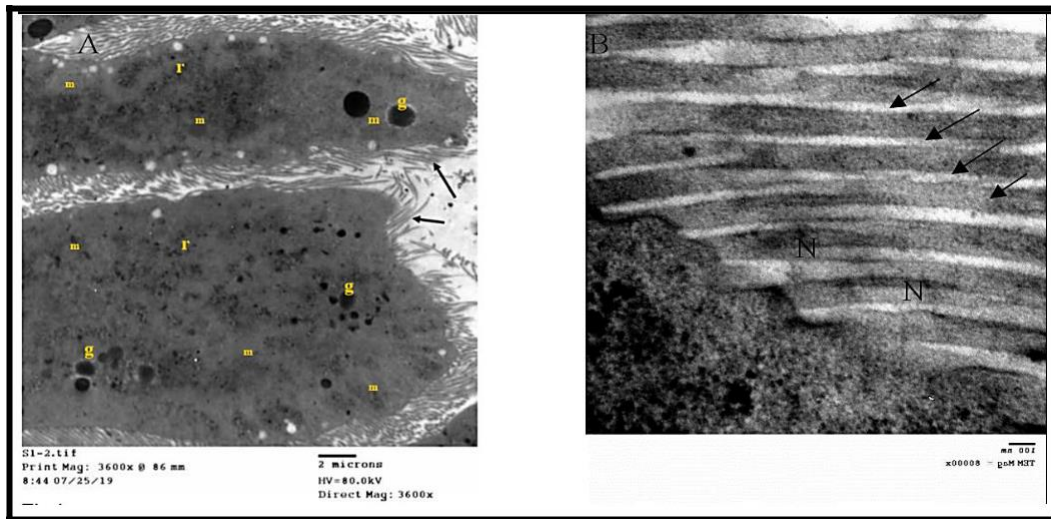


Fig.17 (A&B): TEM micrograph of the untreated larval midgut showing the upper part of the lining epithelia contain numerous electron dens variable size granules (g), clustered free ribosomes (r) and mitochondria (m) .The luminal surface of the cell bearing an apical long regular microvilli border each of obvious nucleus (N). (arrow). [T.B. stain].

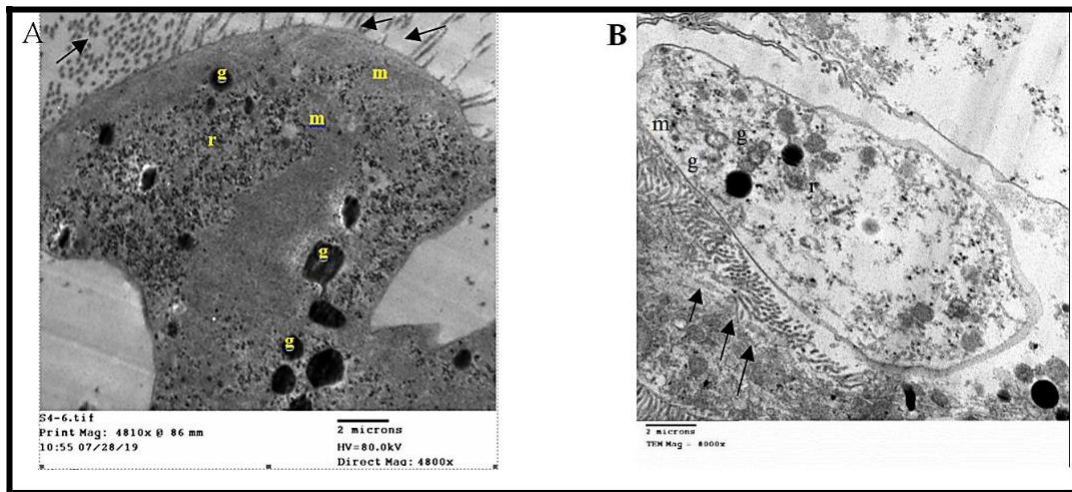


Fig.18 (A&B): TEM micrograph of the epithelial lining of the midgut of *Bt* treated larvae showing its cytoplasm contain numerous large electron dens granules (g), moderate numerous of free ribosomes (r) and swelling of the mitochondria (m) with disintegration of their crest. The luminal surface of the cells having devastating and damaged microvilli variable in length and density (arrow). [T.B. stain].

**NB.**

- 1- The electron dens granules present in the cytoplasm of the cells, mostly fat globules or liposomes spicily the large one and the small one seem to be a secretary digestive enzymes or absorptive material.
- 2- Treated larva showed presence of degenerative changes in the lining absorptive cells and hyperplasia of mucus producing cells (Goblet cells).
- 3- Cellular disintegration in the midgut due to *Bt* toxins exposure has been stated by many authors Castro *et al.* (2019) indicated that the death of caterpillars due to

*Bt* toxins, demonstrated the toxicity of this bacterium through ingestion and a cellular degeneration in the midgut as a result of the toxic effect of *Bt*; Sousa (2010) on *Alabama argillacea*, Ribeiro (2013) on *Plutella xylostella* and Castro *et al.* (2019) on *A.*

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### الملخص العربي

دراسات السمية و البيوكيميائية والخلوية الناشئة على يرقات بكتينوفورا جوسيبيللا (ساندرس) المعاملة بمركب كلوروفلوزيرون وباسيلس ثيرونجينزس

رانيا محمود الشناوي

معهد بحوث وقاية النباتات- مركز البحوث الزراعية- الجيزة- مصر

أجريت تجارب معملية لدراسة تأثير التركيز نصف المميت لمركبي أتابرون و باسيلس ثيرونجينزس على يرقات دودة اللوز القرنفلية بكتينوفورا جوسيبيللا (ساندرس) التي سبق وعولمت كفقس حديث. حيث أظهرت النتائج سمية عالية لمركب ألتابرون مقارنة بمركب الباسيلس. كما أجريت دراسات كيميائية حيوية لتقييم ارتفاع وانخفاض بعض القياسات البيوكيميائية في اليرقات عمر عشرة أيام بعد المعاملة حيث تم قياس كال من نسبة البروتين الكلي الذائب ونسبة الدهون الكلية، الأنزيمات الناقلة لمجموعة المين ALT,AST و كذلك الأنزيمات المحللة للكربوهيدرات (الميليز-النفرتيز- التريهاليز) ، وخلصت النتائج الى انخفاض مستوى البروتين الكلي معنويا الى 43.26 و mg/dl26.24 ( نتيجة المعاملة بمركبي أتابرون وباسيلس ثيرونجينزس على التوالي مقارنة ب) mg/dl100.1 ( لليرقات غير المعاملة وعلى العكس من ذلك، فقد سببت المعاملت ارتفاعا في محتوى الدهون الكلي في اليرقات المعاملة حيث سجل) 132.73 و 87.87 mg/dl ( لنفس المركبين على التوالي مقارنة ب) 36.51 لليرقات غير المعاملة. كذلك لوحظت زيادة في نشاط الأنزيمات المحللة للكربوهيدرات الألفرتيز ، التريهاليز و الأميليز مقارنة بالكنترول. كما أوضحت الدراسة الحالية نقص نشاط انزيم AST في حين زاد نشاط انزيم ALT زيادة ملحوظة ازاء المعاملة بمركبي توبرون وباسيلس وذلك مقارنة باليرقات غير المعاملة.

كما ادت معاملة اليرقات حديثة الفقس لحشرة دودة اللوز القرنفلية بالتركيز نصف المميت لمركب أتابرون على جليد اليرقات المعاملة كأحد مثبطات النمو حيث سببت ظهور تأثيرات هستولوجية واضحة في جدار الجسم خاصة في منطقة الجلد والعضلات حيث أصبحت طبقة الجليد الخارجي اكثر سمكا بينما يقل سمك الطبقة الداخلية بشكل ملحوظ بالضافة الى تحلل طبقة خاليا الغشاء القاعدي وظهور فجوات ،وأوضحت دراسات الميكروسكوب الالكترونى العديد من الفراغات الكبيرة والصغيرة مع وجود اضطراب وفوضى وتليف للنسيج العضلى. بالضافة الى ما سبق، اوضحت الدراسة ظهور تأثيرات هستولوجية واضحة في قناة الهضم لليرقات المعاملة عمر عشرة أيام فظهر في منطقة المعى الوسطى وجود ضعف ملحوظ في طبقة العضلات مع حدوث تهتك حاد و عدم انتظام فى تركيب الخاليا الطائنية العمودية بالضافة لفقدانها للترابط مع احداث تلف في الغشاء المحيط بالغذاء. كما لوحظ حدوث تكون لبعض الفجوات في طبقة الخاليا الطائنية المبطنة للمعى الأوسط لليرقات المعاملة مقارنة بالغير معاملة.