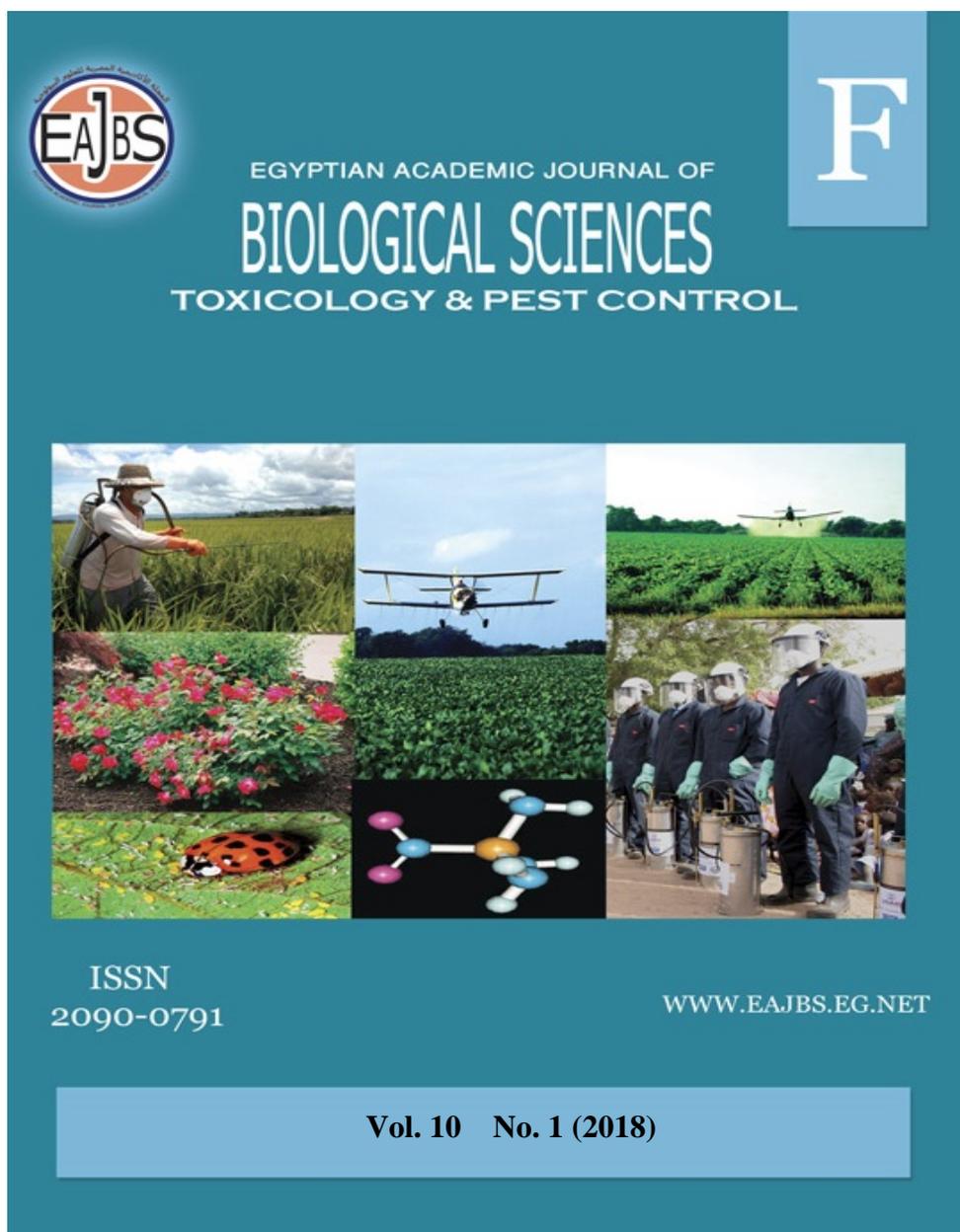


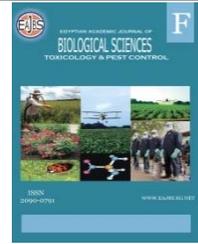
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Role of Teflubenzuron as A Chitin Synthesis Inhibitor Against *Spodoptera littoralis* Larvae

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ABSTRACT

The present research focuses on the effect of teflubenzuron on the 4th larval instar of the cotton leafworm, *Spodoptera littoralis* under laboratory conditions. Teflubenzuron exhibited larvicidal activity (LC₅₀ 8.45 ppm) after 72 hours of treatment. Scanning electron microscopy (SEM) showed growth disruption and abnormalities in the external morphological features of cuticle and head capsule in larvae treated with teflubenzuron. Marked biochemical changes in chitin synthesis pathway were recorded. Protease enzyme activity was significantly reduced, whereas the activities of both chitinase and phenoloxidase were significantly elevated in treated larvae in comparison to controls.

INTRODUCTION

Insect growth regulators (IGRs) represent the third generation insecticides (or biorational insecticides) which are effective as components in integrated pest management (IPM) programs for insect control. IGRs generally control insects by being juvenile hormone agonists, ecdysone agonists, ecdysone antagonists and chitin synthesis inhibitors (CSIs) (Pfeiffer 2007). Two types of CSIs have been developed and used as commercial agricultural pests, benzoylphenyl ureas (BPUs) and buprofezin (Palli and Retnakaran 1999; IRAC 2017). Teflubenzuron is a BPU, (commercial trademark Nomolt). Bažok *et al.*, (2009) reported that teflubenzuron might provide moderate to sufficient level of control in an IPM program against the European corn borer, *Ostrinia nubilalis*.

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is considered as the most important polyphagous pest which attacks many economically important crops through the year (Eppo 1997; Martins *et al.* 2005). The effects of different substituents on the benzene rings in BPUs were analyzed for larvicidal activity against *Spodoptera littoralis* (Nakagawa *et al.*, 1989).

The major objective of the present work is to clarify the role of teflubenzuron as a chitin synthesis inhibitor through examination of the ultrastructure of cuticle and the biochemical changes in *S. littoralis* larvae.

MATERIALS AND METHODS

Chemicals and Reagents:

Teflubenzuron, Nomolt[®] 15 % (SC), *N*-[[[3, 5-dichloro-2, 4-difluorophenyl) amino] carbonyl]-2, 6-difluorobenzamide) was purchased from BASF Co., Germany.

All chemicals and reagents were purchased from Gomhoria Chemical Co., Sharquia Governorate, Egypt.

Rearing Technique of *Spodoptera littoralis*:

A laboratory susceptible strain of *S. littoralis* was used in this study. The strain was reared in the Plant Protection Research Institute at Zagazig, Sharquia Governorate, Egypt according to El-Defrawi *et al.*, (1964). The culture was maintained under optimum conditions (27 °C ± 2 and 70±5 R.H %) and reared on fresh castor bean leaves, *Ricinus communis* until the 4th larval instar.

Bioassay Test:

The leaf-dipping bioassay method was used to determine the median lethal concentration (LC₅₀) value of the tested CSI. Series concentrations of the tested compound were prepared using water (i.e. 75, 37.50, 18.75, 9.37 and 4.68 ppm). Castor bean leaves were dipped for ten seconds in each concentration then left to dry in air. Five replicates (20 newly molted larvae / replicate) were used for each tested concentration as well as control. The treated leaves were offered to newly molted 4th instar larvae for 48 hr then replaced by untreated ones for 24 hr. Mortality percentages were recorded after 72 hr and corrected according to Abbott's formula (1925). To estimate the LC₅₀ value, the corrected mortality percentages were subjected to probit analysis according to Finney (1971). The symptoms of molting failure which leading to larval mortality were examined by Optech light microscope, UK.

Scanning Electron Microscopy Examination:

The external morphological features of cuticle and head capsule of *S. littoralis* 4th instar larvae after 72hr of treatment with LC₅₀ of teflubenzuron as well as the control were observed using SEM. All tested larvae were fixated by glutaraldehyde 2.5 % w/w and dehydrated by serial dilutions of ethanol using automatic tissue processor (Leica EM TP, Germany); then the samples were dried using CO₂ critical point drier (Tousimis Audosamdri-815, Northern Ireland). Samples were coated by gold coater (SPI Module, Clorado USA) and examined by scanning electron microscopy (JEOL-JSM-5500 LV, Tokyo, Japan) using high vacuum mode at Regional Centre of Mycology and Biotechnology, Cairo, Egypt.

Biochemical studies:

Preparation of samples:

The preparation of samples involved the use of newly molted 4th instar larvae of *S. littoralis* after 72 hr of treatment with LC₅₀ of Teflubenzuron as well as control. Ten healthy larvae from each exposure group were picked up and placed in clean jars (Greatglas Inc., Wilmington, USA), then starved for 4 hr. Their tissue (5 mg) was homogenized in distilled water using a chilled glass Teflon tissue homogenizer (ST-2 Mechanic-Preczyina, Poland) inside crushed ice for 3 min. The homogenates were centrifuged at 8000 r.p.m for 15 min at 5 °C in a refrigerated microcentrifuge (Hettich, Germany) to remove haemocytes.

The supernatants were transferred to clean tubes (Greatglas Inc., Wilmington, USA) and stored in freezer at -20 °C until used. Three replicates were used for each biochemical assay.

Total Protein Assessment:

Total protein concentration was assessed according to Bradford, 1976 using bovine serum albumin as a standard.

Enzyme Measurements:

The nomenclature system in enzymes according to Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). The Enzyme Commission (EC) number contains four elements separated by dots. The first number symbolizes the division of enzyme out of six main enzyme groups. The second and third numbers show the subclass and sub-subclass, respectively. The fourth number indicates the serial number of the enzyme in its sub-subclass (Moss, 1992).

- Chitinase (EC 3.2.1.14) activity was determined using 3, 5- dinitrosalicylic acid reagent to determine the free aldehydic groups of hexoamines liberated from chitin digestion according to the method described by Ishaaya and Casida (1974). Absorbance was measured at Xnm (Spectronic 1201, Milton Roy Co., USA).

- Phenoloxidase (EC 1.14.18.1) activity was determined according to a modification of Ishaaya (1971).

- Protease (EC 3.4.21.112) activity was measured according to the method described by Tachell *et al.* (1972) with some modifications: increase in free amino acids released from substrate protein (albumin), after 1 hr incubation at 30 °C was measured at Absorbance was measured at Xnm (Spectronic 1201, Milton Roy Co., USA).

Statistical Analysis:

The data were subjected to statistical analyses by paired t-test to determine the significance of the difference between the treated larvae and control (Snedecor and Cochran 1980).

RESULTS

Toxicological studies:

Teflubenzuron was proven toxic possessing larvicidal activity against 4th instar larvae of *S. littoralis* after 72 hr of treatment. The values of LC₅₀ and LC₉₀ were 8.45 and 49.10 ppm, respectively as shown in Table 1.

Table (1): Toxicity data of teflubenzuron against 4th instar larvae of susceptible strain of *S. littoralis*

Tested CSI	LC ₅₀ ppm (Lower-Upper)	LC ₉₀ ppm (Lower-Upper)	Slope ± SE
Teflubenzuron	8.45 (3.08-11.53)	49.10 (38.52-120.31)	1.70±0.19

Ultrastructure studies:

Different morphological features of molting failure were observed in 4th instar larvae treated with sublethal concentration (LC₅₀) of teflubenzuron using light and scanning electron microscope as illustrated in Figures (1-3).

Normal larvae with successfully molting were recorded in (Figs. 1a & b), while the signs of failure molting leading to mortality were included: Larvae became inactive, appeared to be wet and the molting fluid oozing out the head capsules, (Figs. 1c & d), additionally, partially ecdysed larvae with big batches of new cuticle without normal coloration, (Figs. 1e & f).

The untreated larva has sclerotized head capsule, which dark in color, bears two large epicranials or parietals running antero-dorsally and separated by an inverted "Y" shaped epicranials structures, (Fig. 2a), moreover, in lateral view, the ocellar area is characterized by convexity of its surface and the presence of six stemmata or eyespots, (Fig. 2b). Treated larvae suffered from many marked deformations in its head capsule as it became unsclerotized and lack of "Y" shape, Figures (2c & d), the eye spots may be indistinct and disappeared, (Figure 2c). Further investigations cleared that each side of normal prothorax has a spiracle, which is an external opening of the respiratory system, (Figure 2b), spiracle becomes narrower or even damage in treated larva which leading to suffocation of larvae, (Fig. 2d).

Normal larvae of *S. littoralis* characterized by thoracic segments which connected to the abdomen. Each thoracic segment has a pair of segmented legs assist in locomotion and clinging to substrates, (Fig. 3a), whereas in treated larva, great fissure was recorded between thoracic and abdomen segments, (Fig. 3b). Abnormalities in the external coloration of cuticle was occurred due to incapability of larvae to get rid of the old cuticle (Figs. 3 c & d). Growth disruption in whole body of treated larva that compressed in their old cuticle forming many fissures in between different segments (Figs. 3 e & f).

Biochemical changes:

The changes in the enzyme activities which related to cuticle synthesis of 4th instar larvae of *S. littoralis* as response of treatment with LC₅₀ of teflubenzuron were recorded after 72 hr post treatment in Table (2).

Table (2): Changes in tested enzyme activities of 4th instar larvae treated with teflubenzuron

Treatment	Protease (μg alanine/min/mg protein)	Chitinase (μg alanine/min/mg protein)	Phenoloxidase (O. D. unit/min/mg protein)
Control	64.52 \pm 2.57a	470.13 \pm 8.56a	3069.21 \pm 76.06a
Teflubenzuron	54.31 \pm 1.12b	510.12 \pm 5.70b	3256.48 \pm 137.03b
P	0.022	0.019	0.048
t value	3.63	3.88	3.50

-Each datum represents the mean of three replicates.

-Data expressed as Mean \pm Standard Error (SE).

-Means under each variety having different letters in the same row denote a significant different (P < 0.05).

-Treated larvae at level of LC₅₀ of teflubenzuron.

Protease enzyme:

Teflubenzuron caused significant decrease in protease activity (54.31 \pm 1.12 μg alanine/ min/ mg protein) as compared to control (64.52 \pm 2.57), P= 0.022, (Table 2).

Chitinase enzyme:

The results obtained in Table (2) showed significant increase in the enzyme activity in the larvae treated with teflubenzuron (510.12 \pm 5.70 μg NAGA/ min/ mg protein), while control gave (470.13 \pm 8.56), P= 0.019.

Phenoloxidase enzyme:

Data in Table (2) revealed that teflubenzuron caused significant elevation in phenoloxidase activity (3256.48 \pm 137.03 O.D. unit/ min/ mg protein) comparing with untreated larvae (3069.21 \pm 76.06), P=0.048.

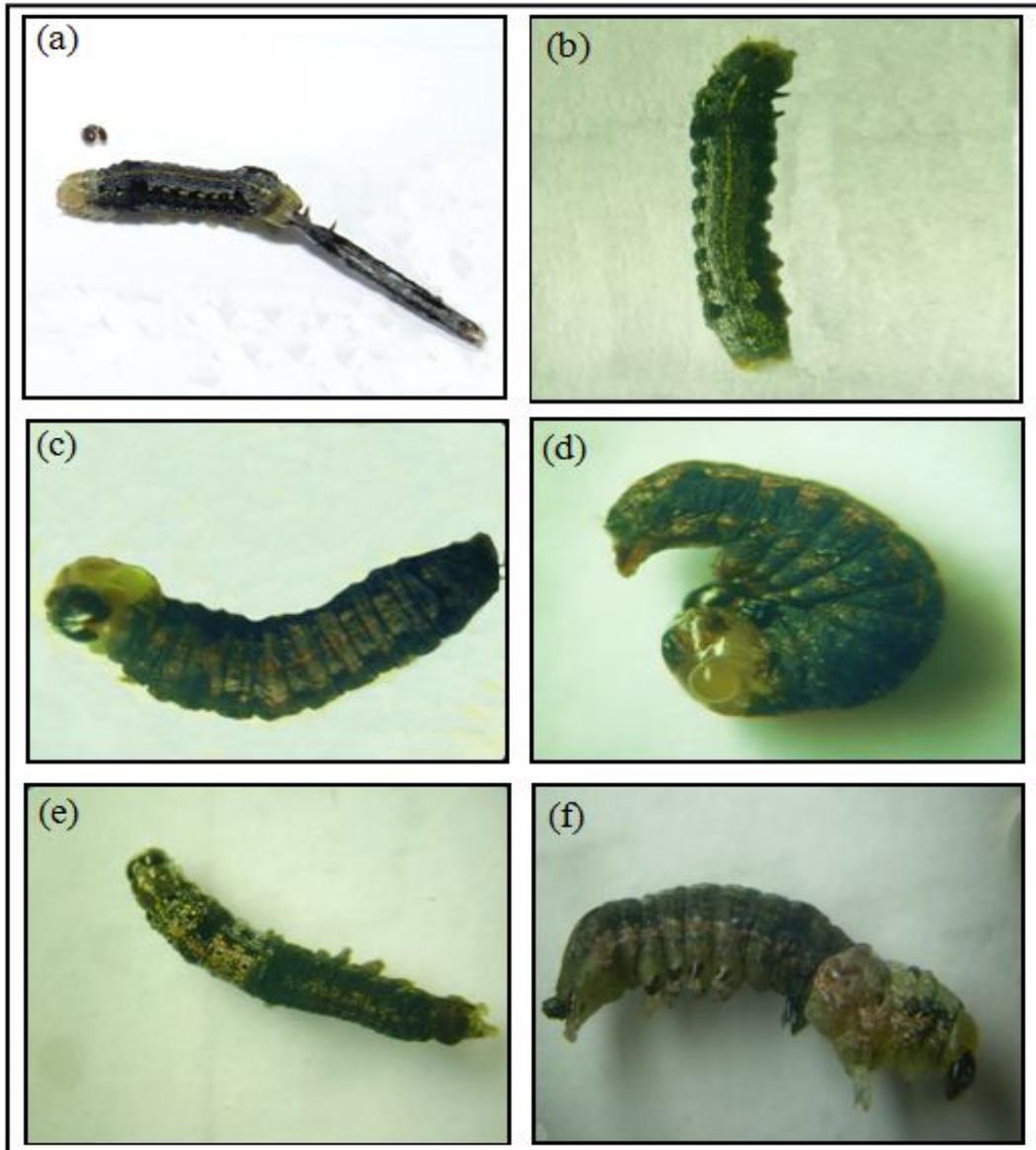


Fig. 1. Photomicrograph of susceptible 4th instar larvae of *S. littoralis* taken 72 hours after treatment showing control larvae (a & b) continue normal growth and development with successively molting. While larvae treated with LC₅₀ concentration of teflubenzuron using ingestion, slipped head capsules, the newly synthesized covering the head is soft, unsclerotized, untanned and malformed (c). Hence molting fluid was hemorrhage from deformed unsclerotized head capsule (d). Additionally, the treated larvae (e & f) initiate a molt, but unable to ongoing the molting process. Thus the intoxicated larvae become moribund and die

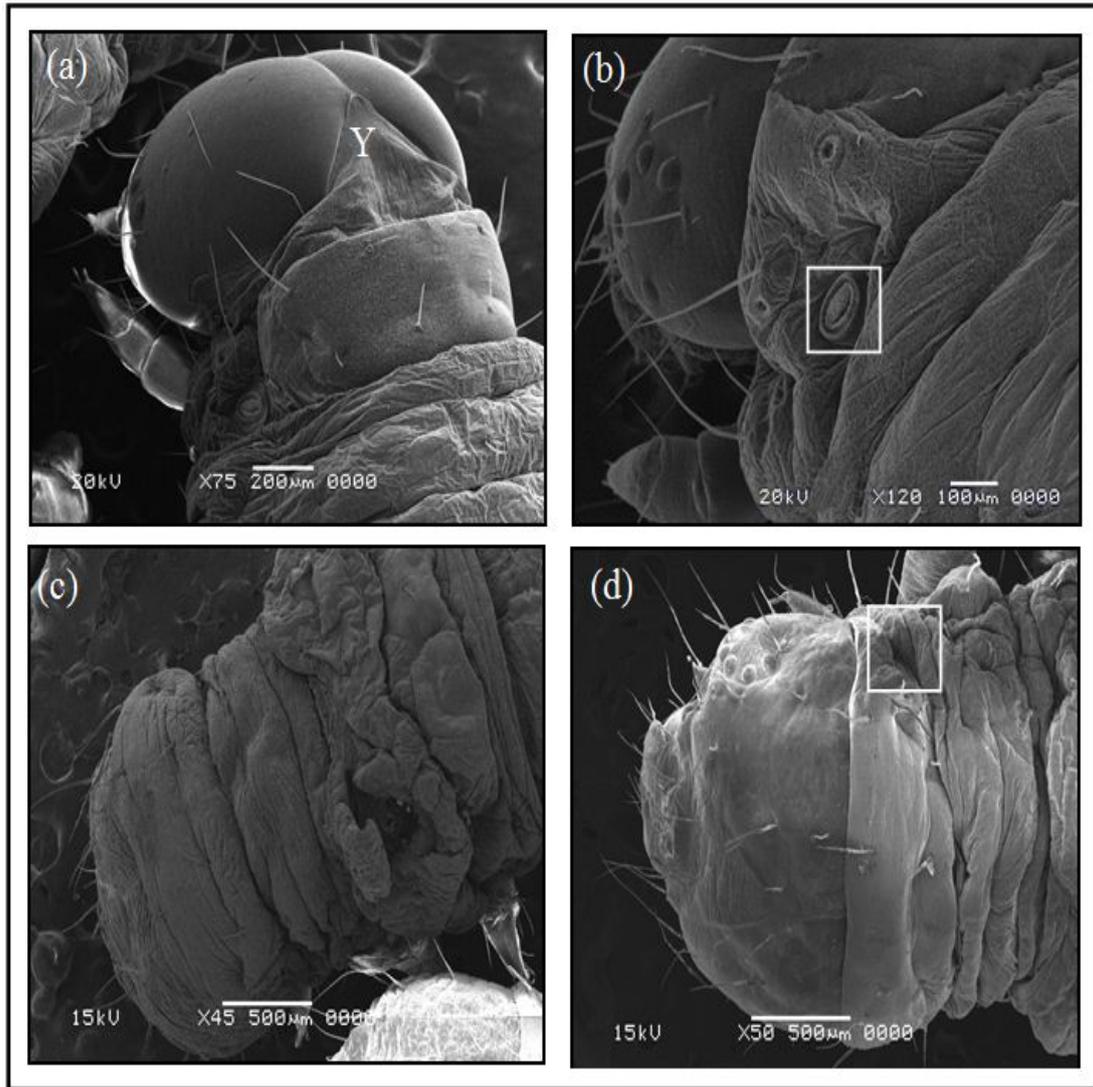


Fig. 2. Scanning electron micrograph showing head capsule and spiracle of susceptible 4th instar larvae of *S. littoralis* after 72 hours of ingestion *R. communis* leaves dipped in LC₅₀ concentration of teflubenzuron. The untreated larvae well formed convex and sclerotized head capsule (a & b), typically contains six stemmata or eyespots and each side of prothorax has a distinguished spiracle (b). The treated larvae slipped head capsule and the unsclerotized new cuticle over the head were formed new cuticle over the head (c & d). Spiracle becomes not clear and more narrower or even damaged, which results in suffocation and ultimate death.

The inner squares (b & d) surround spiracle,

Y: Y shape

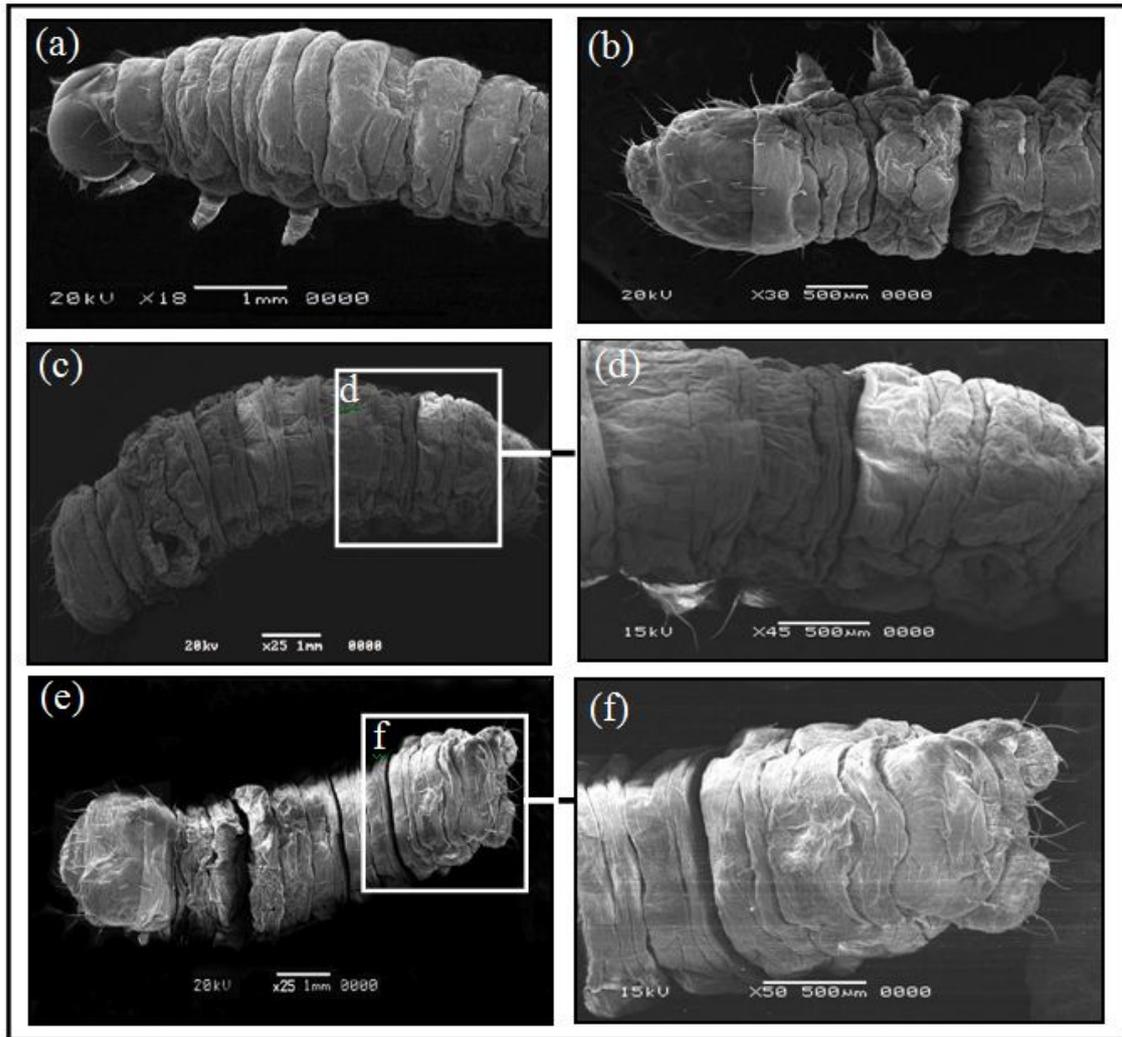


Figure 3. Scanning electron micrograph showing general view of the exoskeleton (cuticle) of the susceptible 4th instar larvae of *S. littoralis* after 72 hours of treatment with LC₅₀ of teflubenzuron. In untreated larva, cuticle is rigid and sclerotized (a). Treated larvae stricken into undergoing the lethal precocious molt. Larva has external fissure between thorax and abdomen segments (b). New chitin precursors were not incorporated into the cuticle, thus larva has Accordion-like structure (c & d). The body of larva was compressed in their old cuticle forming many fissures in between body segments (e & f)

DISCUSSION

In insects, if the cuticle which provides the exoskeletal structure is disrupted during the molting process, this will certainly lead to mortality. This was observed in the present experiment with larvae treated with the LC₅₀ of teflubenzuron. Cuticle must be rigid to provide firm points of muscle attachment as well as for claws and mandibles. Forty-eight hours after ingestion 4th instar larvae of *S. littoralis* on castor bean leaves treated with LC₅₀ of chitin synthesis inhibitor (CSI) or benzophenyl urea (BPU), teflubenzuron, resulted larvae ultimately die as a result of their inability to complete the molting process. As a general trend, BPUs insecticides act on larval stages by inhibiting or blocking the chitin synthesis, a vital and approximately indestructible part of the larvae exoskeleton (Ware and Whitacre 2004). In certain research, when larvae were treated with teflubenzuron, they developed normally until molting, but fail to ecdyse due to inhibition of the synthesis of new cuticle. Sabry and Khedr (2014) reached the same conclusion when they tested IGRs against *S. littoralis* larvae, the molting process was completely derailed at the ultrastructural and biochemical level leading to precocious lethal molt in susceptible larvae.

In our experiments, the intoxicated larvae reveal a lack of sclerotization or tanning of new cuticle or they were unable to discard the old cuticle. In addition, such larvae suffer loss of molting fluid and haemolymph as a result of hemorrhage and damage in spiracle (external opening of the respiratory system). All these lethal symptoms are indicative of the disruption of the ongoing molting process. Similar symptoms were observed by Khedr and Mead (2015) after treatment of *S. littoralis* larvae with tebufenozide and methoxyfenozide. These effects have been demonstrated in a number of lepidopteran larvae treated with tebufenozide (Dhadialla *et al.* 2005).

In this study, teflubenzuron significantly inhibited the activity of protease and significantly elevated the activities of both chitinase and phenol oxidase enzymes as compared to control. These results are similar to that obtained by Sabry and Khedr (2014). Molting fluid contains both chitinase and protease enzymes that digest the main constituents of the old endocuticle (Reynolds and Samuels 1996). Moreover, BPUs affect ecdysone dependent biochemical sites, which lead to chitin inhibition (Zimouska *et al.*, 1994; Oberlander and Silhacek 1998), thus the procuticle of treated larval stages loses its elasticity and the larvae are unable to molt and subsequently die. Accordingly, the changes in these enzyme activities ultimately lead to deformations or mortality of treated larvae. These results were supported by those of Zimowska *et al.*, 1994; Oberlander and Silhacek 1998; Palli and Retnakaran 1999; Oberlander and Samaghe 2001 who found that the mode of action of the BPUs; chlorfluazuron, diflubenzuron and teflubenzuron were include inhibition of chitin synthesis due to an inhibition of the protease enzyme that activates chitin synthase as well as activation of chitinase and phenoloxidase, which are both connected with chitin catabolism.

CONCLUSION

In conclusion, these results show that the larvicidal activity of teflubenzuron against *S. littoralis* was due to abnormalities in the morphological features of the cuticle and marked changes in the chitin synthesis enzymes. This knowledge may be helpful in the control strategies of *S. littoralis*.

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ARABIC SUMMERY

دور تيفلوبنزورون كمثبط تخليق الكيتين على يرقات دودة ورق القطن

هالة محمد ابراهيم ميعاد و محمد محمد أحمد خضر
معهد بحوث وقاية النباتات – مركز البحوث الزراعية – مصر

اهتمت الدراسة الحالية بتأثير مركب تيفلوبنزورون على يرقات العمر الرابع لدودة ورق القطن تحت الظروف المعملية. أظهر مركب تيفلوبنزورون فاعلية ابادية على اليرقات المعاملة بعد ٧٢ ساعة من المعاملة حيث بلغ التركيز القاتل لنصف عدد الأفراد ٨.٤٥ جزء في المليون، و أوضحت الصور الملتقطة بواسطة الميكروسكوب الإلكتروني حدوث اضطرابات في النمو و كذلك تشوهات في التركيب الخارجى لجليد و رأس اليرقات المعاملة. تأثرت العديد من الوظائف البيوكيميائية المهمة لبناء الجليد (الكيتين) تمثلت فى انخفاض معنوى فى نشاط انزيم البروتيز و زيادة معنوية فى نشاط انزيمى كيتينيز و فينول أوكسيديز مقارنة بالكنترول.