



PHYSIOLOGICAL SYMPTOMS INDUCED BY ECDYSONE AGONIST METHOXYFENOZIDE ON THE COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (BOISD.)

[18]

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ABSTRACT

Newly ecdysed fourth instar larvae of the cotton leafworm, *Spodoptera littoralis* (Boisd.) were treated with LC₅₀ of ecdysone agonist methoxyfenozide, to provide better insights into physiological symptoms and aspects induced by it as a mimic to the 20-hydroxyecdysone (20-E) action. Larvae ingest methoxyfenozide died within 2-3 days, and being trapped within their excuvae. They stopped feeding shortly before ecdysis. Treatment neither accelerated ecdysis nor ceased feeding, as soon as the larvae ingest such 20-E mimic. The continuous binding of the compound to the ecdysteroid receptors of *S. littoralis* larvae increased the titre of enzymes regulated by 20-E during the experimental time (3 days). Chitinase and phenoloxidase were activated after 6 and 12 hr from methoxyfenozide administration, respectively. The compound had no effect on chitin production, but endocuticle chitin degradation during moulting might be depressed as illustrated by the presence of higher amount of N-acetylglucosamine in control larvae than methoxyfenozide treated. It is suggested that methoxyfenozide might induces a precocious moult by initiating moulting cycle, but its main toxic effect is due to the fact that its level remains high during moulting and don't decrease for allowing other hormones necessary for successful moulting to be released.

INTRODUCTION

IGR's or chemicals that specifically affect the developmental processes in insects are claimed to

be more ecotoxicological benign than conventional organic insecticides, and they have been successfully used in IPM programs to prevent or delay the rapid development of resistance. Methoxyfenozide belongs to a novel class of IGR's known as diacylhydrazines or nonsteroidal ecdysteroid agonists. It mimics the action of insect moulting hormone, and it is highly selective against lepidopterous larvae (**Palli and Retnakaran 2001**).

Ecdystroid agonists able to induce a premature and lethal larval molt by direct binding to ecdysteroid receptors (**Smaghe et al 2001**). The toxic effect of these compounds is not only due to their hyper-ecdysionism action as they mimic the 20-hydroxyecdysone (20E), but also because their levels do not decrease as 20 E do prior to ecdysis allowing behavioural alterations, and the ecdysis motor program with the production of ecdysis-triggering hormone (ETH) and eclosion hormone (EH), resulting in larval moulting failure and death (**Wing et al 1988; Dhadialla et al 1998; Boudjelida et al 2005**).

The symptoms of poisoning of ecdysteroid agonists have been reported (**Wing et al 1988; Smaghe et al 1997; Smaghe et al 2001; Grafton-Cardwell et al 2005**). In caterpillars, such IGR's force induction of a lethal moulting with cessation of feeding and head capsule slippage coupled with the appearance of an ecdysial space between the epidermis and cuticle in the thorax and the abdomen. A new cuticle may or may not be produced, and the ecdysis process is inhibited so that the intoxicated insect dies within its excuvial cuticle being trapped as pharate insect (**Boudjelida et al 2005**).

Methoxyfenozide use is likely to increase due to its better binding with lepidopteran receptors and longer residuality compared with other di-

acylhydrazines such as tebufenozide (**Grafton-Cardwell et al 2005**). Its toxicity has been studied in the cotton leafworm, *Spodoptera littoralis* as a major polyphagous pest throughout several parts of the world (**Pineda et al 2004; Pineda et al 2006**) and in Egypt.

To provide better insights into methoxyfenozide mode of action on *S. littoralis*, the larvae were treated with LC₅₀ of the ecdysteroid agonist, and we have examined the physiological symptom with the following questions in mind (1) What is the time required after treatment for cessation of feeding as an important symptoms of ecdysteroid agonists? (2) The moulting process starts by apolysis and ends by ecdysis. Does precocious molt caused by methoxyfenozide mean that the entire moulting process is accelerated and the larvae die soon after treatment due to failure of ecdysis? (3) Could starvation, due to cessation of feeding, contributes to larval mortality? (4) What is the effect of treatment on chitin and its degradation product; N-acetylglucosamine? Also the validity of the enzymes that regulated by ecdysone such as chitinase and phenoloxidase as macromolecular symptoms of methoxyfenozide treatment was studied.

MATERIALS AND METHODS

Insects

Newly ecdysed fourth instar larvae of *S. littoralis* were obtained from a continuous stock susceptible colony maintained in the Central Agricultural Pesticides Laboratory (CAPL), Dokki, Giza, Egypt. Larvae were reared throughout the experiments as described by (**El-Defrawi et al (1964)**) under laboratory conditions (25±2°C and 65±5% R/H.).

Chemicals

The dibenzoylhydrazine; methoxyfenozide (24% SC) was obtained from Dow Agrosciences Co., UK. Serial dilutions of the insecticide were prepared in tap water. Other chemicals were of reagent grade and obtained from Sigma (Germany), Merck (Germany) or El-Nasr local Company.

-Ecdysone agonist bioassay

To determine the symptoms induced by LC₅₀ of methoxyfenozide, serial concentrations were prepared in water. Freshly collected castor bean leaves were dipped for 20 seconds in each con-

centration, then left one hour to dry. Newly ecdysed fourth instar larvae (4-10 hr) were confined with tested leaves in glass jars covered with muslin cloth for 48 hr. Five replicates each of which had 10 larvae were tested for each concentration. Control larvae were fed on water-tested leaves. Survived larvae were fed on untreated leaves for another 24 hr. The mortality percentages were recorded after 72 hr from treatment, and corrected as compared to control larvae according to Abbott formula (**Abbott, 1925**). To estimate LC₅₀ value, the corrected mortality percentages were subjected to probit analysis according to the method of **Finney (1952)**.

Larvae treated with LC₅₀ were used to describe some characteristic symptoms of methoxyfenozide poisoning. Weight gain, as indicator to cessation of feeding, was recorded at 24, 48 and 72 hr after treatment. Cuticle enzymes, N-acetylglucosamine and chitin content were examined at 6, 12, 24, 48, 60, 66 and 72 hr.

-Gravimetric ex vivo determination of chitin

The dissected body integuments were air dried and weighed to evaluate their fresh weight. Chitin content was determined as described by **Berghiche et al (2007)**. Integuments were washed for 24 hr in ether-chloroform (1:1, v/v) and dried at 60°C. Then they were treated with NaOH (2 N) at 100°C for 2 hr to remove proteins. The residue obtained was considered to be chitin and was rinsed quickly with ethanol, air dried and weighed. The chitin content was referred to the original fresh weight of integuments.

-Sample preparation for biochemical assays

For the determination of N-acetylglucosamine and the enzymatic activities, the larvae were starved for about 4 hr before being homogenized. Three to five larvae were rinsed with normal saline and homogenized in 2 ml distilled water in a glass tissue grinder under an ice jacket. The homogenate was centrifuged at 8,000 rpm for 20 min at 4°C to remove cellular and mitochondrial debris. The supernatant is collected and stored at -10°C until further use.

-Measurement of N-acetylglucosamine

N-acetylglucosamine (NAGA) measured using modified Ehrlich reagent (1 gm p-dimethylaminobenzaldehyde dissolved in 50 ml of

glacial acetic acid and 2.5 ml conc. HCl) by the method of **Waterhosue et al (1961)**. NAGA content was expressed as $\mu\text{g NAGA mg}^{-1}$ tissue.

- Chitinase activity assay

Colloidal chitin prepared as described by **Bade and Stinson (1981)**, was used as the substrate for chitinase activity. The reaction mixture consisted of 100 μl phosphate buffer (0.1 M, pH 7), 100 μl colloidal chitin and 100 μl enzyme solution, and incubated at 35°C for 2 hr. the enzyme activity was terminated by boiling test tubes for 10 min.

NAGA produced from chitin digestion was estimated as mentioned before. A standard linear plot of absorbance versus amount of NAGA was obtained. Chitinase activity is expressed as $\mu\text{g NAGA mg}^{-1}$ tissue min^{-1} .

-Phenoloxidase activity assay

Phenoloxidase activity was determined with aqueous catechol as substrate (**Ishaaya, 1971**) at the prestablished conditions. The reaction mixture that consisted of 1 ml phosphate buffer (pH 7, 0.1 M), 200 μl catechol (3%), was incubated at 40°C for 5 min, and then enzyme solution was added to initiate the reaction. The absorbancy at 405 nm was recorded for 2 min. phenoxidase activity was expressed as $\Delta\text{OD}_{405} \text{ mg}^{-1}$ tissue min^{-1} .

-Statistics

All the obtained values were pooled from triplicate (at least $n = 10$) and are presented as the mean \pm SD. Colorimetric determinations were repeated 3-5 times. The dose-response data were submitted to probit analysis, and the lethal concentration (LC) was calculated in ppm (95% CL). Data were subjected to ANOVA and the least significant difference were used to separate means, and then they were compared by multiple range tests ($P < 0.05$) from the Costat program.

RESULTS AND DISCUSSION

Since many insecticides show secondary actions at high concentrations (**Van Eck 1979**), the median lethal concentration (LC_{50}) was used throughout the present experiments where physiological symptoms were expected to be evident. Methoxyfenozide was ingested by newly ecdysed fourth instar larvae of *S. littoralis* for 48 hr and the LC_{50} value was calculated after 72 hr (**Fig. 1**). It

was 1.19 ppm (95% CL = 1.02-1.38 ppm; slope = 1.50).

Treatment by methoxyfenozide as an ecdysone agonist, gave an impression that treated cotton leafworm would suffer from two symptoms. The first is the larvae would undergo moulting process, and die due to failure in completing this process, at a time more shorter than the normal control, i.e. treatment would shorten the duration of the instar which lasts about 60 hr (**Amin, 1998**). The second is the weight gain of the treated larvae would be decreased as a result of feeding cessation, which characterizes the treatment by such compounds.

Indeed, the treated larvae did not show any ecdysis failure during the first 24 hr after treatment (**Table, 1**). After 48 hr, 7% of the larvae died and trapped within their exuvium. The highest mortality (= 52%) was recorded after 72 hr were elapsed. Close observations revealed that body color changes appeared in those affected larvae after 48 hr from treatment including cessation of feeding and production of brown color with white patches, then it converted to black color. On the other hand, treatment had significant effect on the larval duration after treatment and up to the pupal stage (**Table, 1**). Larval duration was 184 and 228 hr for control and treated larvae, respectively. It seems that methoxyfenozide might led moulting process to start precociously, but the time for its ending with ecdysis is not accelerated.

Cessation of feeding was not observed, at least for most larvae, during the first 48 hr after ingestion of LC_{50} of methoxyfenozide. This emphasized by the measured larval weight (**Fig. 2**). There were only significant changes after 72 hr, whereas the larval weight for treated and control was 153 and 250 mg/larva, respectively.

Due to its an ecdysonergic activity and/or continuous binding to the ecdysteroid receptors, methoxyfenozide induced some macromolecular symptoms in the cotton leafworm larvae that fed on LC_{50} of this toxicant. These symptoms were detected by the presence of high titres of enzymes regulated by ecdysone in a time differed from that occur in normal larvae. After 12 hr from treatment, Phenoloxidase titre (**Fig. 3**) began to increase more than that of control, it was 2.46 and 1.58 $\Delta\text{OD}_{405} \text{ mg}^{-1}$ tissue min^{-1} for treated and control larvae, respectively. After 48 hr, Phenoloxidase activity sharply increased in normal larvae indicating that moulting period was due (continues about 12 hr), then the titre began to decline reaching its lowest level after 72 hr. On the other hand, in

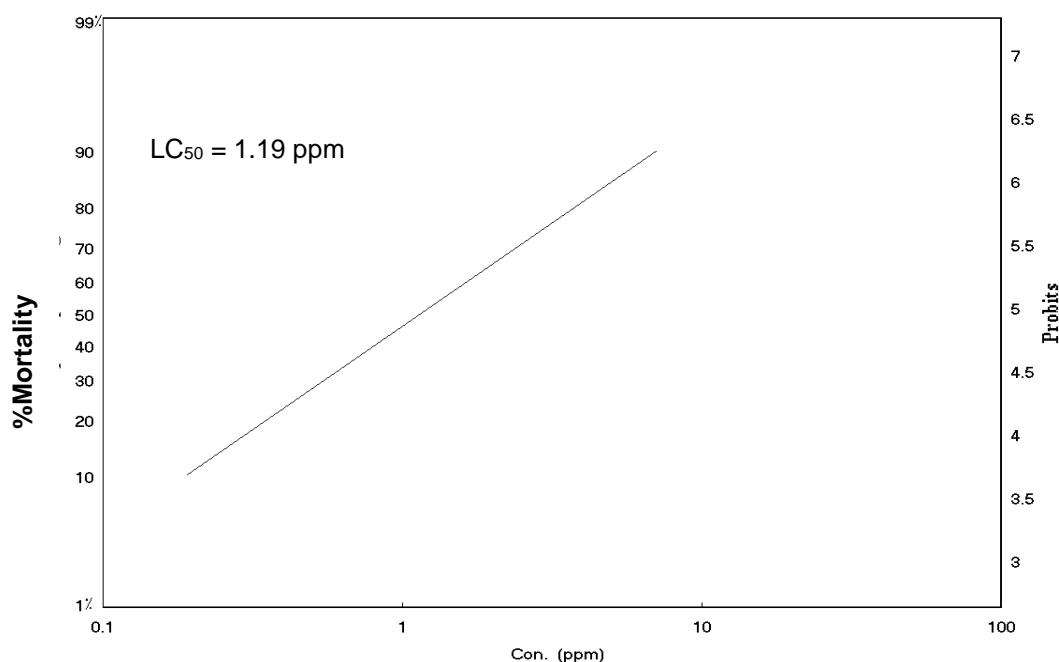


Fig. 1. Concentration-mortality regression line of the 4th larval instar of *Spodoptera littoralis* after 72 hr from treatment with the ecdysone agonist; methoxyfenozide

Table 1. Percentage mortality and larval duration of *Spodoptera littoralis* after treating fourth instar larvae with LC₅₀ of the ecdysone agonist; methoxyfenozide

Diet	%Mortality			Larval duration (hr)
	24 hr	48 hr	72 hr	
Control	0.00	1±0.5	3±1	184±5 ^a
Treated	0.00	7±1	52±3	228±6 ^b

-Time = hours after treatment

- Data are presented as the mean±SD

-Means bearing the same subscripts are non significantly different ($P < 0.05$, ANOVA, LSD test)

treated larvae the titre of the enzyme was higher than control during the time of experiment, except during moulting period. The same trend was observed for chitinase activity (Fig. 4). However, the increased level of the chitinolytic enzyme was detected after 6 hr from treatment.

The results from Table (2) revealed that the overall value of chitin content relative to fresh weight for 72 hr period was 2.58 ± 0.66 for the control group and 2.38 ± 0.89 for the treated group. An analysis of variance and Duncan's multiple range analysis demonstrated no significant differences

between the treated and control larvae in the level of chitin. However, chitin content after 48 hr from treatment for control larvae was greatly decreased than treated ones. This finding was encountered by high level of NAGA at the same period with respect to control larvae (Table 2), while during the intermoult period (lasts about 48 hr from ecdysis), NAGA was at its basal level in both control and treated larvae.

As normal larvae enter the moulting stage that consists of a definite sequence of events initiated by the moulting hormone or ecdysone and includes

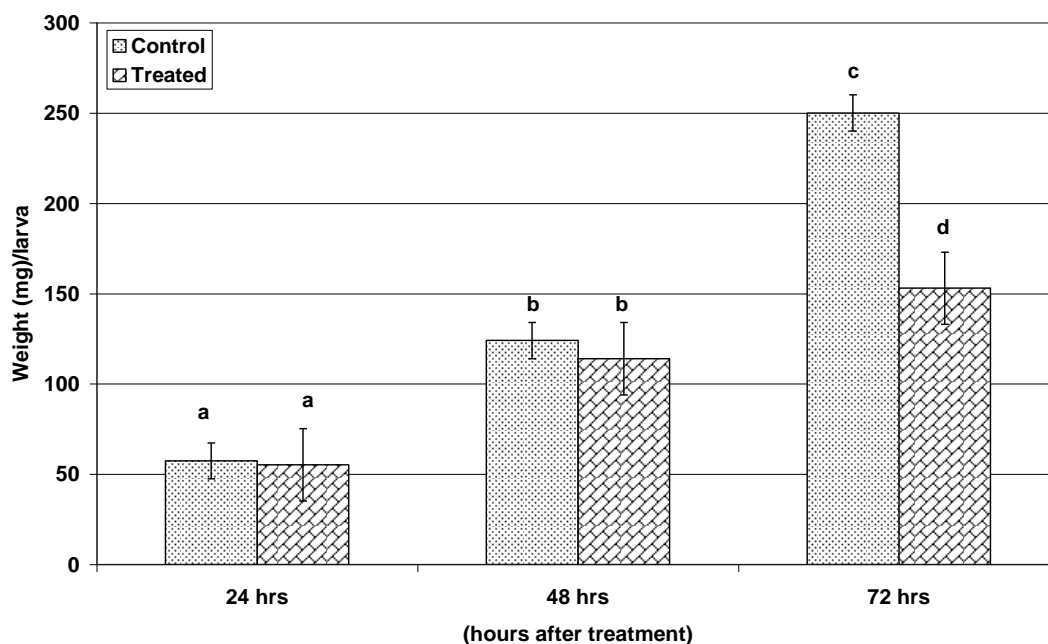


Fig. 2. Weight of *Spodoptera littoralis* larvae after ingestion of fourth instar to LC₅₀ of methoxyfenozide

-Means bearing the same subscripts are non significantly different (P < 0.05, ANOVA, LSD test)

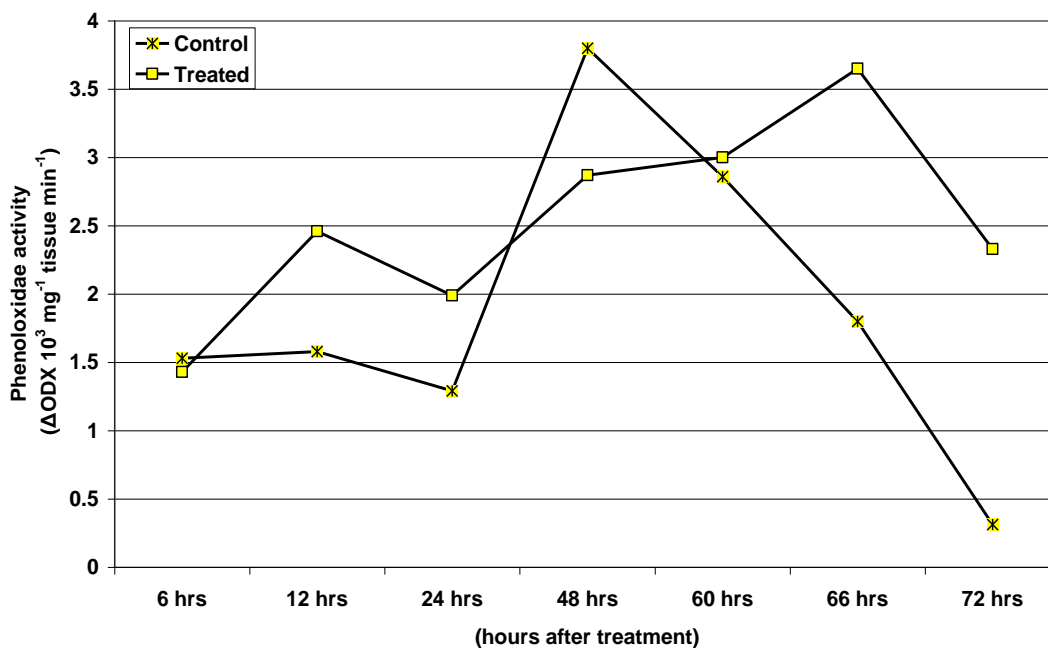


Fig. 3. Phenoloxidase activity after treatment of newly ecdysed fourth larval instar of *Spodoptera littoralis* by LC₅₀ of methoxyfenozide

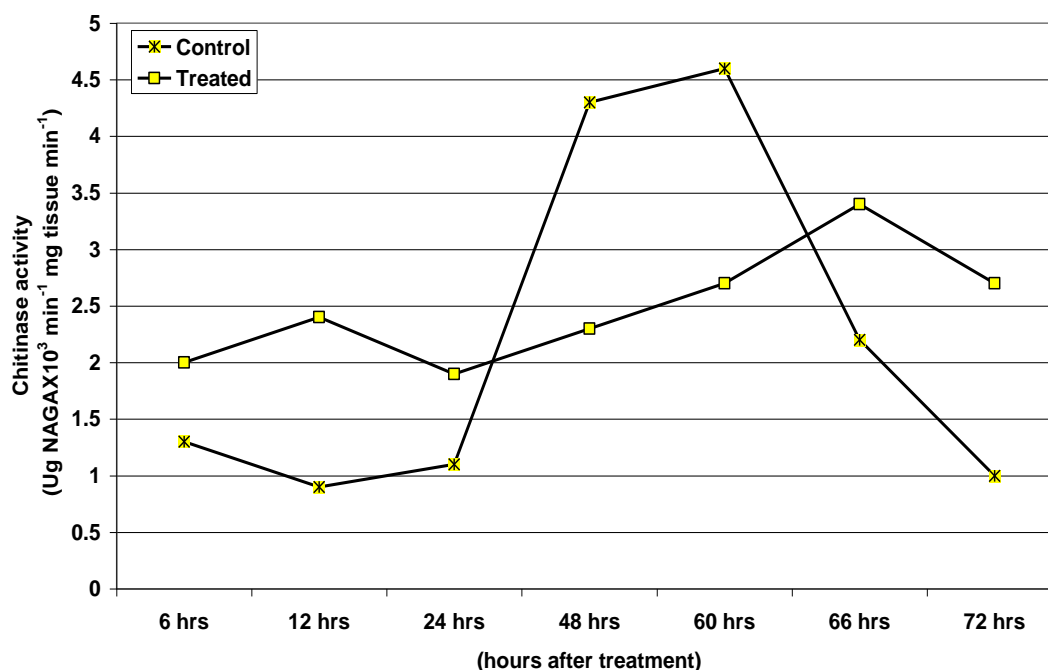


Fig. 4. Chitinase activity after treatment of newly ecdysed fourth larval instar of *Spodoptera littoralis* by LC₅₀ of methoxyfenozide

Table 2. Chitin and N-acetylglucosamine content of newly ecdysed fourth instar larvae of *Spodoptera littoralis* treated with LC₅₀ of methoxyfenozide

Hours after treatment	Chitin content relative to fresh integument (W/W,%)		NAGA content (ugX10 ³ mg ⁻¹ tissue)	
	Control	Treated	Control	Treated
12	-	-	35±6 ^a	26±4 ^a
24	1.32±0.21 ^a	1.58±0.19 ^a	25±3 ^a	30±5 ^a
48	1.79±0.13 ^a	2.4±0.25 ^b	349±12 ^a	135±10 ^b
66	3.12±0.3 ^a	2.99±0.27 ^a	156±2 ^a	170±2 ^a
72	3.3±0.11 ^a	3.1±0.22 ^a	29±4 ^a	49±3 ^a

- Data are presented as the mean±SD

- Means, within a row, bearing different subscripts are significantly different (P< 0.05, ANOVA, LSD test)

- n = 6 integuments for experiment of chitin determination, and n = 10 larvae for NAGA content.

a definite sequence of events: apolysis, moulting fluid production, initiation of new cuticle formation, moulting fluid activation, excuticle secretion and ecdysis, they stop feeding till completion of moulting process (**Chapman, 1982**). Cessation of feeding as characteristic symptom of lepidopteran insects poisoning by ecdysone agonists had been reported. **Smaghe et al (1997)** reported that tomato lopper larvae treated with tebufenozide could suffer gut alterations, suggesting that such larvae stopped feeding. **Grafton-Cardwell et al (2005)** also reported that lepidopteran larvae stop feeding within a few hours from tebufenozide treatment. Based on the results of the present experiments, we found that when newly ecdysed fourth instar larvae of *S. littoralis* ingested LC₅₀ of methoxyfenozide, they did not immediately stop feeding, but this occurred shortly before ecdysis. This suggests that feeding cessation symptoms that accompany moulting period, not occurs due to high titre of ecdysone or its mimics such as methoxyfenozide, but when the insect at the physiological level that ensures completion of moulting process by ecdysis.

The decrease of weight gain observed in the present study, have been reported in several caterpillars (**Pineda et al 2006**). Excluding the possibility that methoxyfenozide act as a potent feeding deterrent, since the larvae, during the present experiments were observed to eat their food more or less as control, methoxyfenozide might causes gut alterations to the treated insects as reported by **Smaghe et al (1997)**. On the other hand, there are some reports that rely upon dehydration and/or starvation as the causes of larval death after moulting disruption as a result of treatment by ecdysone agonists. The present results revealed that the larvae did not suffer from starvation during 48 hr after treatment, but their weight become reduced about 40% after 72 hr. **Ebeling (1971)** suggests that insect death by dehydration occurs usually when about 30% of the initial weight has been lost. Besides, **Amin (1998)** found that starved fourth instar larvae could withstand starvation for several days without death. So it suggested that dehydration might be the main reason of larval death due ecdysis failure after methoxyfenozide treatment.

Grafton-Cardwell et al (2005) reported that tebufenozide is a dibenzoylhydrazine stomach poison that acts as IGR specifically for lepidopteran. The treated insects undergo a premature lethal moult within 3 to 7 days. Authors agree that treatment by such IGR's induces a precocious and le-

thal larval moult (**Wing et al 1988; Smaghe et al 2001; Boudjelida et al 2005**). Also, the present results revealed that highest percent of ecdysis failure and death occurs after 72 hr from treatment of *S. littoralis* newly ecdysed fourth instar with LC₅₀ of methoxyfenozide. So it is suggested that the ecdysone agonist might initiates moulting process including apolysis, without accelerating the entire sequents that ends by ecdysis failure and death, specially the moult cycle depends on several factors. **Locke (1974)** has suggested that the developmental sequence is linked at the genetic level in the epidermal cells and that ecdysone functions as the initial stimulus with other stimuli (both extrinsic and intrinsic) acting at later stages to control quantitatively the expression of individual phases of sequences.

A correlation between the rise in ecdysteroid titre, and the increase in activity of chitin-degrading enzymes and cuticle tanning enzymes has been shown in various arthropods (**Rees 1977; Koga et al 1991**). Treatment of *S. littoralis* larvae by methoxyfenozide led chitinase and phenoxidase activity to be higher than control (**Figs 3&4**), except during the moulting period (48 hr after treatment) where the titre of the enzymes in normal larvae was higher than that of treated larvae. This means that treatment by the ecdysone agonist induces high titres of chitinase and phenoxidase like ecdysone in normal larvae. Thus, the induction of chitinase and phenoxidase activity after treatment could be considerable as physiological symptoms of methoxyfenozide poisoning. Also, the continuous high activity of both enzymes during the experimental time in the treated larvae indicates the continuous binding of the insecticide to the ecdysteroid receptors in the larvae, confirming that ecdysteroid toxic action not only due to it mimic 20 E, but also due its level don't decrease at basal level to ensure successful moult.

Various studies had shown that ecdysteroid agonist compounds were able to induce the incorporation of [¹⁴C] GLCNAC for a higher chitin deposition (**Oikawa et al 1993**). As shown in **Table (2)** methoxyfenozide had non significant effect, *in vivo*, on chitin production in the integuments of the cotton leafworm. **Berghiche et al (2007)** found that the chitin of integumental explants of *Tenebrio molitor* was increased significantly only with RH-0345 and RH-2485. Methoxyfenozide significantly increased chitin content of treated larvae more than control during moulting period, while it decreased NAGA content of the same larvae (**Table 2**). This might indicates that methoxyfenozide

inhibit chitin degradation during molting process, consequently NAGA content was lesser in the treated larvae. This emphasized by the observed decreased titer of chitinase in methoxyfenozide ingested larvae as compared to control (Fig. 4).

Based on the results presented, it can be suggested that methoxyfenozide might induces a precocious moult by initiating moulting cycle of the larvae after treatment, but its main toxic effect is due to the fact that its level remains high during moulting period and don't decrease for allowing other hormones necessary for successful moulting to be released. Finally, methoxyfenozide has probable potential in formulating novel IGR-based control tools against *S. littoralis* larvae in environmental friendly manner to the ecosystem.

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بعض الظواهر الفسيولوجية الناتجة من معاملة يرقات دودة ورق القطن بمبيد الميثوكسيفينوزيد

[18]

طارق رئيس أمين¹ - عزيزة حسن محمدي²
1- معهد بحوث وقاية النباتات - مركز البحوث الزراعية - الدقي - الجيزة
2- المعمل المركزي للمبيدات - مركز البحوث الزراعية - الدقي - الجيزة

الملخص العربي

التجارب أن المركب لا يسرع من عملية الإنسلاخ. وقد حفزت المعاملة نشاط إنزيمي الكيتينيز والفينول أوكسيديز وذلك بمجرد المعاملة وحتى نهاية التجربة (بعد 3 أيام). كما إتضح أن المركب لا يؤثر علي تكوين الجليد ولكنه ربما يؤثر علي تحلل طبقة الكيوتيكال الداخلية أثناء عملية الإنسلاخ. لقد رجح من خلال هذه الدراسة أن تأثير المركب السام ليس لكونه يحاكي هرمون الإنسلاخ فقط ولكن لثبات المركب داخل الحشرة الذي يؤدي الي عدم ظهور الهرمونات المرتبطة بإنخفاض مستوي هرمون الإنسلاخ والهامة لنجاح عملية الإنسلاخ .

تم معاملة يرقات العمر الرابع لدودة ورق القطن المنسلخة حديثاً بمبيد الميثوكسيفينوزيد الذي يعمل عن طريق محاكاة هرمون الإنسلاخ وذلك بغرض دراسة بعض الظواهر الفسيولوجية وذلك لفهم طريقة عمل هذا المركب. تم تغذية اليرقات علي ورق خروع معاملة بطريقة الغمر وذلك بالتركيز النصف قاتل من هذا المبيد (ج ق50). أظهرت النتائج أن نسب الموت تحدث في الفترة ما بين 2-3 أيام بعد المعاملة وكانت اليرقات ميتة ومحاطة بجلد الإنسلاخ. وقد بدأت الإمتناع عن الأكل قبل الإنسلاخ بساعات. كذلك دلت

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