Laboratory Study on Some Potential Histological Changes Induced by The Toxicity of a Mixed Insecticide (spinetoram + methoxyfenozide) on The Spiny Bollworm, *Earias insulana*

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Abstract

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The present laboratory study was conducted using the larvae of spiny bollworm, *Earias insulana* (Boisd.) (Lepidoptera: Nolidae) to observe the correlation between the toxicity effect and the potential histopathological changes induced by feeding with LC_{50} of the pesticideUphold (36% SC), a commercial insecticide mixture formulation of two active ingredients [spinetoram 6% + methoxyfenozide 30% (w/v)]. The microscopic observations showed morphological variations in the midgut and fat body that indicated a defect in the performance of their functions. The treated larvae showed modifications in the midgut tissues vs the control sections, whereas the epithelial cells seamed without any distinct stratification and had malformed morphology along the midgut. The peritrophic membrane lining loss was evident. The abdominal fat body of the treated larvae appeared in densely spread lobes, accumulating and occupying an enormous portion of the abdominal cavity. Its cellular components were characterized by their large size compared to the untreated ones. The results obtained indicated that the tested mixed insecticide gained its properties from its active ingredients. The findings herein indicated that chronic exposure to the applied insecticide could be considered stressful conditions affecting different aspects of fat body functions needed for late vital activities. That effect could be viewed as a possible disruptive impact on the detoxification mechanism and biosynthetic-fat body functions required during the advanced stages ofthe development and maturation of the treated larvae.

Keywords: Spinetoram, Methoxyfinozide, Spiny bollworm, *Earias insulana*, Midgut, Fat body.

Introduction

Cotton plants (*Gossypium barbadense* L.) are targeted by many insect pests, and the spiny bollworms *Earias insulana* (Boisd.) (Nolidae, Lepidoptera) are among the most dangerous pests on cotton plants in Egypt (Abul-Nasr *et al.*, 1972). This pest is present on cotton plants at the beginning of the growing season and flower formations. It is highly active from mid to late cotton season (Amer *et al.*, 2015). It feeds on terminal buds and flowers. Its activity and intensity increase with the appearance of bolls on plants. In Egypt, several pesticides are recommended to control the cotton bollworm complex, *Pectinophora gossypiella* and *E. insulana*. Also, many experiments were conducted annually to develop recommended insecticides safer for human health and the environment, and to overcome the ability of the bio-system of the pest to detoxify and resist the toxic effect. Using new types of pesticides from natural agents or products that disrupt the target pest's physiological processes could be helpful as alternatives in the integrated management approaches (Dhadialla *et al.*, 1998; Smagghe *et al.*, 2003). Methoxyfenozide 30% is one of the main members of the IGR (insect growth regulators) group that mimic the action of the steroid insect moulting hormone, which induces premature and incomplete moulting, resulting in larval mortality (Dhadialla *et al.*, 1998). Against lepidopterous pests, methoxyfenozide has been widely influential, and there are many reports on its efficacy in different crops. (Moulton *et al.* 2002; Pineda *et al.* 2004; Smagghe *et al.* 2003; Sparks *et al.*, 1998; Thompson *et al.*, 2000; Williams *et al.* 2004). As mentioned by Ahmed *et al.* (2022), numerous studies on lepidopterous pests in several regions have demonstrated resistance to methoxyfenozide. Several studies have highlighted that using a methoxyfenozide in a mixture with spinosyn class insecticide such as spinetoram showed a more positive effect on the biological properties of the targeted lepidopterous pests under field conditions compared to its use alone (Shobharani *et al.*, 2019). Ahmed *et al.* (2022) documented that methoxyfenozide +spinetoram mixtures are effective against methoxyfenozide-resistant *S. littoralis* strains and can be used in IPM programs. Uphold (36% SC), is a formulated mixture of two insecticides containing two active ingredients (spinetoram + methoxyfenozide). The Agricultural Pesticide Committee, Egyptian Ministry of Agriculture recommended using this mixed insecticide in tomato fields against cotton leafworm *S. littoralis*. *E. insulana* larvae are considered the predominant pest at the beginning of the cotton season and damage both buds and bolls. Moreover, it becomes a severe pest of cotton in the mid and late seasons when most plants have already started producing bolls, necessitating a decision to treat with pesticides. The current study aimed to correlate the toxicity effect with potential histopathological changes on the midgut and fat body of the *E. insulana* larvae fed on a mixed insecticide (spinetoram + methoxyfenozide) (Uphold 36% SC) under laboratory conditions.

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Materials and Methods

Insect Rearing

E. insulana, a laboratory strain, was obtained from a colony maintained in the Bollworms Research Department, Plant Protection Research Institute, Agriculture Research Centre. Larvae were reared on an artificial diet as described by Amer (2015) and incubated at 26±1°C. The pest rearing and bioassay tests were carried out under the same conditions. The pest colony had no history of insecticide exposure. Adults were fed with a 10% sugar solution under the same condition and were allowed to lay their eggs on tissue paper as an oviposition substrate which was replaced periodically.

Chemical insecticides

The product tested was Uphold (36% SC), a commercial mixed insecticide formulation of two active ingredients: spinetoram 6% + methoxyfenozide 30% (w/v). Both ingredients were provided by DOW Agro Sciences. Methoxyfenozide is an insect growth regulator and an excellent pest control option for integrated pest management (IPM). Spinetoram is an insecticide mixture of two active neurotoxic constituents of *Saccharopoly spinosa* (Bacci *et al.*, 2016).

Toxicity of the insecticide by larval feeding

The LC_{50} of the tested compound against *E. insulana* larvae was estimated by preparing halfway series of six concentrations ranging between 4.5 and 0.14 ppm using tap water. Each concentration was tested by preparing three replicates of glass tubes (2×7 cm) (30 sterile tubes/replicate), each containing approximately 3.0 gm of an artificial diet prepared according to Amer (2015). A drop of 0.2 ml per each prepared concentration was spread on the upper surface of the artificial food's tubular content using a dropper. In addition, three replicates were prepared with the same method, replacing the drops of the insecticide concentrations with drops of water to serve as a control. In both treated and untreated replicates, a single newly hatched larva was seeded in each tube with a camel hair brush, and each tube was tightly closed with a cotton stopper. All treatments were incubated at a temperature of 26±1°C. Larval mortality was recorded 24 hrs after treatment. A larva was considered dead if no movement was observed. Based on the Abbott formula (Abbott, 1925), mortality was calculated and subjected to probit analysis, according to Finney (1971).

Histological procedures

The estimated LC50 value through the toxicity assessment previously explained was used to correlate the toxic effect with potential histopathological changes on the midgut and fat body of the *E. insulana* larvae. As previously mentioned, three replicates of glass tubes (30 sterile tubes /replicate), each containing 2.0 grams of artificial diet, were performed. A drop (0.2ml) of the LC50 was spread on the upper surface of the artificial food's tubular content. In addition, three replicates were prepared in the same manner, replacing the drops of the insecticide concentrate with drops of water, as control. Newly hatched *E. insulana* larvae were seeded individually in each treated and control tube. The treated and control treatments were kept for ten days by following the above-described exposure procedures and insect conditions described for the larval toxicity bioassay. Live larval samples (ten larvae) from both treated and untreated were used for histological studies. The larvae were anaesthetized with ethyl ether. Treated and untreated larvae were fixed (10 h) in Bouin's fluid after cutting off their heads and end for fixation, dehydrated in ethyl alcohol concentration series and cleared in xylene. The samples were then embedded in soft and hard paraffin and cut into 6µ thick sections before staining. Sections were stained with Ehrlich's haematoxylin and counter-stained with eosin (Culling, 1974) and observed under a light microscope at 400x magnification, and photomicrographs were taken.

Results and Discussion

The lethal concentration (LC_{50}) value was determined using six halfway serial concentrations of mixed spinetoram + methoxifenozide) insecticide against newly hatched *E. insulana* larvae. From the Probit analysis, LC_{50} of the tested insecticide was estimated at 1.09 ppm, slope ± SE = 2.161 ±0.170. In the present study, the microscopic examination of the control midgut sections (Figure 1-A, 1-B and 1-C) showed that the epithelial layer of midgut tissue appeared as a corrugate wall composed of three primary cell types: columnar, goblet, and regenerative. The columnar cells emerged mostly as giant cells in the midgut epithelium. These cells have grooved edges or delicate villi. Each cell had an oval central nucleus, and the goblet cells have a distal opening and an oval nucleus. The structure of midgut wall of the control had similar characteristics to those described previously by Lehane & Billingsley (1996) and Sousa *et al.* (2009). Such studies showed that in lepidopteran larvae, the midgut epithelium has four primary cells: columnar and goblet, and there are two types of cells, regenerative and endocrine, which are localized at the base of the midgut epithelium to ensure the growth and regeneration of cells of the midgut to keep pace with growth and development. In this study we did not detect endocrine cells of the midgut epithelium. They were difficult to observe under conventional histological staining techniques, similar to those reported in earlier studies (Endo & Nishiitsutsuji-Uwo, 1982; Endo *et al.*, 1983; Lehane & Billingsley, 1996). The microscopic observations of the treated *E. insulana* larvae (Figure 1-D, 1-E and 1-F) showed a range of changes in the midgut tissues compared to the control sections, columnar, regenerative and goblet cells seamed without any distinct stratification, and the peritrophic membrane lining loss was evident. Other regions showed the midgut epithelial in a discrete shape from the peritrophic membrane lining, columnar cells were more delicate and lengthy, and a significant increase in microvilli-sites at some lining points was noticed.

On the other hand, previous studies documented that the fat body consists of a mass of cells underneath the epidermis. In some insects, the fat body also surrounds the digestive and reproductive organs (Cakici, 2017; Han & Bordereau, 1982; Thomsen & Thomsen, 1974; Martins *et al.*, 2011). Histologically, the fatty body varies significantly

according to the different orders of insects. However, in the same species, the histological composition of these cells is consistent (Dean *et al.,* 1985). In the current study, the untreated *E. insulana* larvae histological fat body features showed general views of the abdominal fat body (Figure 2-A, 2-B and 2-C). The results obtained showed that the abdominal fat body is distributed under the integument and organized in lobes of different thicknesses and can be seen either in direct contact with abdominal organs or separated by a muscular layer.

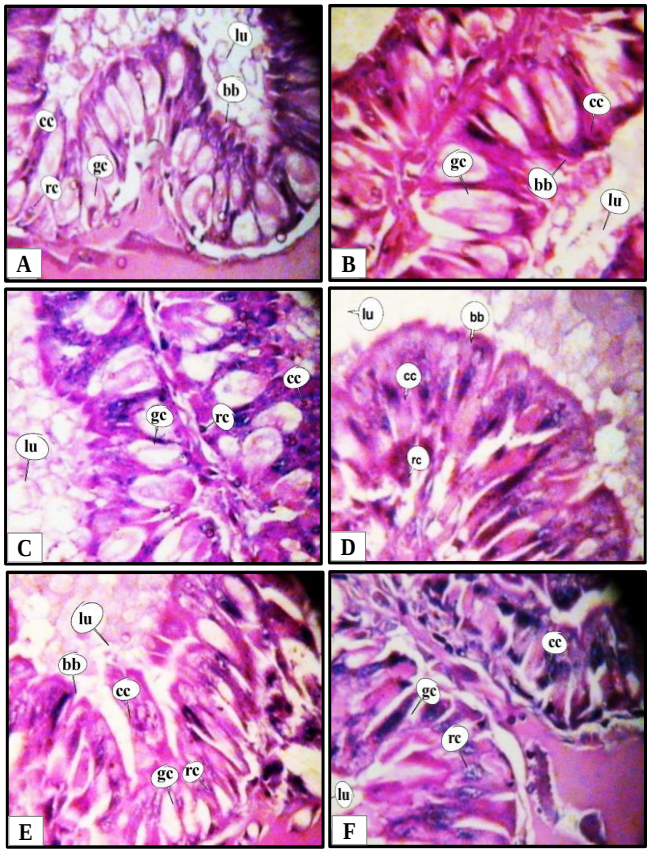

Figure 1. Histological aspects of mid-gut epithelium in *E.insulana* untreated larvae vs treated with spinetoram + methoxifenozide insecticides mixture. A, B and C: the untreated mid-gut epithelium showed that the epithelial layer of midgut tissue appears as a corrugate wall composed of three distinct primary cell types: columnar cell (cc), goblet cell (gc), regenerative cell (rc), and the brush border (bb) were visible facing the gut lumen (lu). In most sections, goblet cells have emerged as giant cells in the midgut epithelium of untreated larvae. D, E and F: the microscopic observations of the treated *E. insulana* larvae showed modifications in the mid-gut tissues compared to the control sections. Columnar cells looked most prevalent in the different regions of the midgut epithelium compared to the goblet cells. The midgut epithelial cells seamed without any distinct stratification. It was noticed that in the treated tissues, the adhesion of cells to each other has faded, creating spaces between them, the brush border appeared intermittent, and the regenerative cells looked massive.

The cells appeared as organized spheroid with vacuoles and pleomorphic nuclei. Morphologically (Figure 2-D, 2-E and 2-F) the fat body of the treated larvae appeared in densely spread lobes and accumulated in a uniform shape, showing a spherical shape and occupying a large portion of the abdominal cavity. Its cellular components were characterized by their largeness compared to the untreated.

It is necessary to study the effect of insecticide on the organelles of the insect to understand the impact a toxicant product, especially if the immature stages of insects are treated with a mixture of more than one insecticide to correlate the toxicological effect with potential histopathological changes. Our results showed that the mixed insecticide (spinetoram + methoxyfinozide) could induce histomorphological changes simultaneously in multiple organs, like what it did to the midgut and fat body of *E. insulana*-treated larvae (Figure 1 and 2).

Several studies reported ways to overcome pests' resistance to pesticides, including mixing pesticides with different modes of action (Ahmad *et al.*, 2009; Attique *et al.*, 2006). Even though spentoram + methoxyfenozide, both of which have different effects on insects, the histological results obtained in this study on the midgut agree with several earlier reports that showed similar effects to spinosyns the action of compound on the target insects' midgut. Many studies have reported that the spinosyns group (spinosad, spinetoram) showed more effect on the insects when used in a mixture with an insect growth regulator than when applied individually (Shobharani *et al.*, 2019). Ahmed *et al.* (2022) have shown the power of the pesticide mixing effect (spinetoram + methoxyfenozide) on the cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae). They have documented a significant decrease in the activity of the esterase enzymes vs no change in the activity of that enzymes when using the mixture components individually. They suggested that the effectiveness of this mixture can be attributed to the fact that mixing increases the ability of both compounds when combined enhances the effect of disrupting the metabolic pathway of insect detoxification. Our observation showed that the epithelial cells (columnar, goblet and regenerative) of the treated *E. insulana* larvae had malformed morphology along the midgut. It was considered that the spinosyns group act on insects through the effects of nicotinic acetylcholine andγ-aminobutyric acid (GABA) receptors causing abnormal neural transmission and death (Shimokawatoko *et al.*, 2012). Effects on the midgut tissues of the treated larvae in this study indicated that there may be different additive toxicological mechanisms regarding the toxic effects of this insecticide. That may be because the insect midgut is the primary site of the insecticide entry point into the insect body, and poisonous molecules first cause alterations in that organ to affect insect physiology (Fiaz *et al.*, 2018; 2019; Santos Junior *et al.*, 2020). This study confirm what has been reported by Perez- Perez *et al.* 2014 who suggested that spinosad induces autophagy in midgut digestive cells. They suggested that there may be different toxicity mechanisms targeting the non-nerve-cell concerning the neurotoxic effects of this insecticide. The misshapen tissues of the midgut resulting from the treatment in the current study may lead to organ dysfunction.

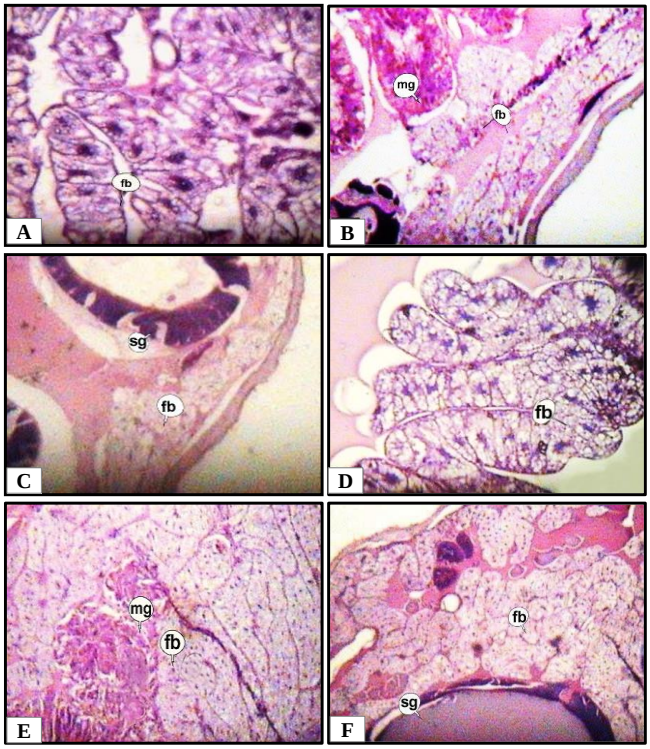

Figure 2. A, B and C: the untreated *E. insulana* larvae's histological fat body features showed that the fat body was organized in lobes of different thicknesses. The cells appeared as organized spheroid vacuoles. D, E and F: the fat body of the treated larvae with mixed insecticides spinetoram + methoxifenozide morphologically appeared in densely spread lobes and crammed and occupied a large portion of the abdominal cavity with cellular components marked by their massiveness as compared to the untreated.

It may impair enzyme secretion and nutrient absorption (Nasiruddin & Mordue, 1993). The insecticide, in the form of a mixture (spinetoram + methoxyfenozide) may be characterized by its higher lethal effect against insects compared to applying them individually (Warnock & Cloyd, 2005). Our results showed simultaneous histological changes in multiple organs and the midgut.

The fat body of *E. insulana*-treated larvae appeared in a non-normal shape in densely spread lobes, accumulating and occupying a large portion of the abdominal cavity. Its cellular components showed distinction by their largeness compared to the untreated. Due to its importance, several studies have described the function of the fat body in insects to be more or less similar to that of the liver in vertebrates (Hoshizaki, 2005; Liu *et al.*, 2009). Alamer (2013) mentioned that the fat body synthesizes and stores lipids, glycogen, free carbohydrates and proteins, and other metabolites. Like other larval organs, the fat body is a target tissue for the action of all principal hormones of the insects (LaFont, 2000). Fat body development and function are regulated mainly by neural hormones, juvenile hormones and ecdysteroids (Keeley, 1985; Liu *et al.*, 2009). One fat body function related to ecdysteroids is synthesis and secretion of proteins in the haemolymph. The insect fat body synthesizes and secretes amounts of different proteins: storage proteins, utilized as an amino acid reservoir for morphogenesis; lipophorins, which are dependable for the transport of lipids in the haemolymph; vitellogenins, used during egg development in females (Keeley, 1985). Limited data is available about the effect of spinetoram and ecdysone agonists on histological changes of the fat body of Lepidoptera. Methoxyfenozide is a prominent member of the IGR group that mimics the action of ecdysteroid hormones in Lepidoptera (Alamer, 2013; Wing *et al.*, 1988). Ecdysteroids activate and stimulate the fat body's uptake of the storage and transport proteins.

20-hydroxyecdysone (20E) plays an essential role in this process (Burmester & Scheller, 1995; Ismail & Dutta-Gupta, 1990; Tojo *et al.*, 1982; Ueno *et al.*, 1983). Maiza *et al.*, 2004, stated that an IGR (methoprene) caused an increase in ovarian protein content, which led to the rise in the size of basal oocytes in German cockroaches. Methoxyfenozide is an ecdysteroid agonist and can disrupt ecdysteroid-regulated functions in insects. We suggest that this may explain the dense spread and the massive size of the fat body in the treated larvae, whereas the methoxyfenozide has a role in confusing hormonal control and disturbs the balance of protein accumulation in the fat body. These results indicate that the tested mixed pesticide gained its properties from characteristics of its active ingredients; spinetoram as the fast-acting toxic ingredient as a member of the spinosyns group -causing abnormal neural transmission and death- (Shimokawatoko *et al.*, 2012) and physiological toxicity represented by methoxyfenozide as an ecdysteroid agonist -disrupt ecdysteroid-regulated events in insects- (Pineda *et al.*, 2006). The results obtained in this study suggest that these properties distinguished the tested pesticide and explained its long-acting effect compared to what is known about the properties of each active ingredient alone. Based on the function of the fat body and its action as a biosynthetic intermediate (Giorgi & Mazzini,1986; Haunerland & Shirk, 1995; Haunerland *et al.*, 1990; Wuest, 1978) and its key role in the development of insects (Skowronek, 2021), the findings presented here indicate that chronic exposure induced by the applied insecticide, could be considered stressful conditions affecting different aspects of fat body functions needed for late vital activities. That effect could be viewed as a possible disruptive impact on the detoxification mechanism and biosynthetic-fat body functions required during the advanced stages of the development and maturation of the treated larvae.

الملخص

أشرف، فايز أحمد، مرفت عبد السميع قنديل ومصطفى عبد الحكيم محمد شلبي. 2023. دراسة مختبرية على بعض التغييرات النسيجية المحتملة الناتجة عن سمية خليط المبيد الحشري (سبينتورام + ميثواكسيفينوزايد) على دودة اللوز الشوكية (*Earias insulana*). مجلة وقاية النبات العربية، 41(4): 437–443. https://doi.org/10.22268/AJPP-41.4.437443

في هذه الدراسة المختبرية، تم استخدام الطور اليرقي لآفة دودة اللوز الشوكية، وهي من عائلة حرشفية الاجنحة، لربط التأثير السمي بتشوهات نسيجية محتملة قد تنتج عن التغذية على بيئة غذائية صناعية معاملة بالجرعة النصفية المميتة (LC_{50}) بعد تقديرها (1.09 جزء في المليون) للمبيد الحشري ابهولد (36% SC) وهو مبيد حشري تجاري مخلوط من مكونين من المواد الفعالة (سبينتورام + ميثوكسيفينوزايد). أظهرت نتائج الفحص المجهري للأنسجة وجود اختلافات شكلية في المعي الأوسط والجسم الدهني في اليرقات المعاملة مشيرة إلى وجود خلل في أداء وظائفهما. أظهرت اليرقات المعالجة وجود تغيرات في أنسجة المعي الأوسط مقارنة بغير المعاملة، حيث بدت الخلايا الظهارية بدون معالم مميزة ومشوهة، كما كان التشكل الخلوي على طول المعي الأوسط مع فقدان بطانة الغشاء المحيطي. ظهر الجسم الدهني البطني فى اليرقات المعاملة بفصوص كثيفة الانتشار ومتراكمة وتحتل جزءاً كبيراً من تجويف البطن، تميزت مكوناته الخلوية بضخامتها مقارنة بالمكونات غير المعاملة. أشارت الدراسة النسيجية الحالية إلى أن يرقات دودة اللوز الشوكية التي تمت معالجتها قد أظهرت تغيرات نسيجية متزامنة في أكثر من عضو واحد (المعي الأوسط والجسم الدهني)، وعانت من سمية مزمنة ناتجة عن التغذية على المبيد المختلط، والذي تسبب بالإضافة إلى ذلك في سمية حادة لليرقات حديثة الفقس تحت إجراءات التقييم الحيوى للمبيد. أشارت هذه النتائج أيضاً إلى أن المبيد المختلط المختبر اكتسب خصائصه من خصائص مكوناته الفعالة. ميّزت هذه الخصائص المبيد الذي تم اختباره بأنه مديد المفعول مقارنة بما هو معروف عن خصائص كل مكون فعال على حدة. كما أشارت النتائج النسيجية، وبخاصةٍ على الجسم الدهني، إلى وجود تأثير يوحي بوجود إرباك محتمل في آلية إزالة السموم والتخليق الحيوي ذات الصلة بالجسم الدهني خلال المراحل المتقدمة من تطور ونضج اليرقات المعاملة.

كلمات مفتاحية: سبينيتورام، ميثوكسيفينوزايد، دودة اللوز الشوكية، *Earias insulana*، المعي الأوسط، جسم دهني.

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