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Effect of Pre-Treatment and Histological Alterations Induced by Sub Lethal Dose of Lufenuron on Both Chlorpyrifos and Imidacloprid Efficacy on Cotton Leaf Worm, *Spodoptera littoralis* (Bosid.)

Noura A. Hassan ; M. Morshedy and N. Shaker
Faculty of Agriculture - Alexandria University - El Shatby

ABSTRACT

Chlorpyrifos and Imidacloprid are belongs to two main groups of insecticides that are widely used in insect control. Many researches had been discussed the effect of both groups on non-target organisms and the environment. So, it necessary to reduce the used doses of this groups to save the environment. The effect of pre-treatment with lufenuron (chitin synthesis inhibitor) at sub lethal dose (LD_{25}) on the efficacy of Chlorpyrifos and Imidacloprid on *Spodoptera littoralis* was studied using topical application method. The toxicity of both Chlorpyrifos and Imidacloprid increased after 24hrs of pre-treatment with LD_{25} of Lufenuron by 55 and 250 times for Chlorpyrifos and Imidacloprid, respectively compared with the toxicity of each compound alone. Histological studies showed that the treatment of 2nd instar larvae with LD_{25} of Lufenuron caused obvious separation of the hypodermis layer from the endocuticle, abnormalities in the epicuticle morphology , rupture in muscles, irregular and great disintegration of cuticle and hypodermis. In-vivo interaction of LD_{25} values of Chlorpyrifos, Imidacloprid and Lufenuron with Alkaline and Acid phosphatase caused significant increase in the activity of Alkaline phosphatase while Acid phosphatase activity was significantly decreased in all treatment.

INTRODUCTION

The cotton leaf worm, *Spodoptera littoralis* (Bosid.) (Lepidoptera: Noctuidae), is a polyphagous species attacking several crops (e.g., cabbage, soybeans, cotton, and other vegetables and cash crops) and causing worldwide lossess (Xue *et al.* 2010).

However, the extensive and continuous use of traditional insecticides creates environmental contamination and could lead to development of insect resistance. Different protocols have been used to reduce pesticides risks to be safer for human health and the environment (Wing *et al*., 2000; McKinley *et al*., 2002). Numerous institutions have extensively implemented alternative methods such as use of insect growth regulators (IGRs) including juvenile hormone analogues (JHAs), chitin synthesis inhibitors (CSIs) and ecdysteroids (Post and Vincent, 1973; Slama, 1974; Staal, 1975; El-Ibrashy, 1970; Tunaz and Uygun, 2004).

On the other side to the classical chemical insecticides and biolarvicides, IGRs are not directly toxic, but act selectively on the growth, metamorphosis or/and reproduction of the target species (Hoffmann and Lorenz 1998, Martins & Silva 2004).

IGRs are ‘‘low risk’’ insecticides, which have a relatively minor detrimental effect on the environment and its inhabitants, making them as an important component in IPM programs (Horowitz and Ishaaya, 2004).

Among IGRs, the chitin synthesis inhibitors (CSIs) act by interfering with the synthesis or deposition of chitin on the exoskeleton or other chitinised internal structures, such as the peritrophic matrix (Merzendorf and Zimoch 2003, Merzendorf 2005).

Benzoylphenylureas (BPUs) which are thought to be chitin synthesis inhibitors, belong to a class of insect growth regulators (IGRs) that has been proposed as alternative insect control agents (Graf,1993). They appear to act as inhibitors of a yet undefined step(s) in chitin synthesis pathway, disrupting moulting at critical developmental stages of insects (Hajjar,1979; Fournet, *et al*,1995). Benzoyl Phenyl Ureas (BPUs) are considered safe insecticides for humans and other mammals, although chitin is absent in these species (Apperson, *et al*, 1978).

Early applications of synergism (Busvine, 1971) revealed that a mixture of chemicals with different mode of action can result in more potent use of toxicants that could theoretically inhibit or delay the emergence of resistant strains. In this respect, organophosphates synergize/ pyrethroids were used against several pests, i.e., S. littoralis (Asher *et al*., 1986), Heliothis armigera (Martin *et al*., 2003). Further, synergism was obtained by combinations of IGRs and traditional insecticides (El-Guindy *et al*., 1985; Radwan *et al*., 1985, 1990; Kandil *et al*., 2006).

The present work aimed to investigate the effect of pre-treatment of sub-lethal dose of Lufenuron on the efficacy of Chlorpyrifos and Imidacloprid against 2nd instar larvae of Spodoptera littoralis. Histological parameters and alkaline and acid phosphatase activities as biochemical parameter were studied .

MATERIALS AND METHODS

Insect:

Laboratory strain of cotton leaf worm, Spodoptera littoralis was used in this studies, the strain was obtained from Medical Entomology lab, Applied Entomology Dept, Faculty of Agriculture, Alexandria University. Insect was maintained under laboratory temperature of 27±2°C and 65±5 RH.

Tested compounds:

A. Imidacloprid

Technical grade compound 80% :(E) -1- (6-chloro-3-pyridylmethyl) - N- nitroimidazolidin – 2 -ylideneamine. (Neonicotinoids group)

B. Chlorpyrifos

Technical grade compound 97%: O, O diethyl O 3, 5, 6 trichloro -2- pyridyl phosphorothioate. (Organophosphates group)

C. Lufenuron (Chitin synthesis inhibitor)

Technical grade compound 94% : 1 - [2,5 - Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy) phenyl] -3-(2,6-difluorobenzoyl)urea.

Bioassays and treatments:

The toxicity of technical tested insecticides was evaluated against 2nd instar larvae of S.littoralis lab strain by topical application method. Series of doses of the tested insecticides were prepared in acetone, 10 larvae in each replicate, and three replicates for each concentration. One microliter acetone solution of the tested insecticides was topically applied to the whole dorsum of each larvae using Arnold hand micro-applicator (Burkard, Rickmansworth Herts, England).

An acetone treated control was included in each experiment. The treated larvae were transferred to clean Petri dishes with pieces of fresh castor oil leaves. LD50 values were recorded after 24 and 48 after larval exposure. The data were corrected using Abbott's formula (Abbott, 1925), and the mortality percentage were analyzed using log-probit program (Finney, 1971).

The toxicity of Chlorpyrifos and Imidacloprid was carried out as mentioned before using pretreated larvae with LD_{25} of Lufenuron after 24hrs.

Biochemical studies

Preparation of samples:

The survival larvae after 24hrs of treatment with LD50 of Chlorpyrifos, Imidacloprid and Lufenuron were collected and homogenized in distilled water using a Teflon homogenizer surrounded with a jacket of crushed ice for 3 minutes. Homogenates were centrifuged at 6000 r.p.m. for 10 minutes at 5°C. the supernatant was used for protein , alkaline and acid phosphatase activity determination.

Assay of phosphatase activity.

Both alkaline phosphatase (ALP) and acid phosphatase (ACP) were determined according to Bessey *et al* 1946 using sod- p- nitro phenyl phosphate as substrate. AIP and ACP activities were calculated as µmoles of liberated p- nitro phenol per minute per mg protein. The concentration of p- nitro phenol is calculated from the standard curve.

Determination of total protein:

Total protein was determined according to lowry, 1951.

Histological studies

For the histological studies, 2nd instar larvae were treated by the values of LD25 of Lufenuron was applied by topical application method then the living larvae after 24hrs were used for this study which carried out at the Electro-microscopic unit- Faculty of Science- Alexandria University.

Statistical analysis

Data were analyzed by one-way analysis of variance and Student Newman Keuls (S.N.K) test at $p<0.05$ using SPSS (version 16.0; SPSS Inc. Chicago, IL, USA).

RESULTS

Toxicity tests.

The results for the toxicity of the three tested compounds are listed in Table (1), the LD50 values after 24hrs were 0.0055 , 0.25 and 9 µg/larvae for Chlorpyrifos , Imidacloprid and Lufenuron ,respectively. And after 48hrs, the LD50 values were 0.0008, 0.037 and 0.9 µg/larvae for Chlorpyrifos and Imidacloprid and Lufenuron , respectively.

The toxicity of Chlorpyrifos alone and after pre-treatment with LD25 of Lufenuron by 24hrs compared against second instar larvae of cotton leaf worm are shown in Table (2) which clearly indicated that the toxicity of chlorpyrifos after pre-treatment with Lufenuron is 55times more than that of Chlorpyrifos alone.

The toxicity of Imidacloprid after pre-treatment with LD25 of Lufenuron by 24hrs compared with the toxicity of Imidacloprid alone against second instar larvae of cotton leaf worm are shown in the same Table which clearly increased the toxicity of Imidacloprid after pre-treatment with Lufenuron is 250times more than that of Imidacloprid alone.

Table 1. LD_{50} values of tested compounds against 2nd instar larvae of *Spodopteera littoralis* (Bosid) after 24 and48 hrs.

Treatments	LD_{50}^* µg/la (24hrs)	95% confidence Limits		Slop±SE	LD_{50}^* µg/la (48hrs)	95% confidence Limits		Slop±SE
		Lower	Upper			Lower	Upper	
Chlorpyrifos	0.0055	0.0025	0.0129	0.9162±0.024	0.0008	0.0006	0.0014	0.9166±0.0833
Imidacloprid	0.25	0.1223	0.622	0.5705±0.072	0.037	0.0249	0.0579	0.7887±0.0741
Lufenuoron	9	7.1204	11.69	.9038±0.2647	0.9	0.649	1.219	0.9014±0.0834

LD_{50}^*: Lethal dose that cause 50% mortality

Table 2. LD_{50} values of Chlorpyrifos and Imidacloprid against 2nd instar larvae of *Spodopteera littoralis* Biosd after 24 pre-treatment with LD_{25} value of Lufenuron.

Treatments	LD_{50}^* µg/la	95% confidence Limits		Slop±SE
		Lower	Upper	
Chlorpyrifos alone after 24hr	0.0055	0.0025	0.0129	0.9162±0.024
Chlorpyrifos after 24hr pre-treatment with Lufenuron	0.0001	0.0001	0.0002	0.6777±0.0900
Imidacloprid alone after 24hr	0.25	0.1223	0.622	0.5705±0.0722
Imidacloprid after 24hr pre-treatment with Lufenuron	0.001	0.0008	0.0018	0.7861±0.0980

LD_{50}^*: Lethal dose that cause 50% mortality

***In-vivo* interaction of tested compounds with Acid & Alkaline phosphatase.**

The results for in-vivo interaction between the LD50 values of tested compounds with Acid and Alkaline phosphatase are recorded in Table (3), the results reported significant inhibition of acid phosphatase for all treatments compared with the control but no significant differences between all

treatments (Chlorpyrifos, Imidaloprid and Lufenuron). While all treatments caused significant increase to alkaline phosphatase activity of the enzyme compared to the control .Lufenuron and Imidacloprid have very low increase effect, while Chlorpyrifos caused the most significant increase in the activity (71.11%).

Histological studies

The normal integument as known is consist of three main layers starts from the inside to the outside are, the basement membrane , the hypodermis which is the only living portion in the integument then the third layer, the cuticle which consist of three parts, endo,exo and epicuticle .

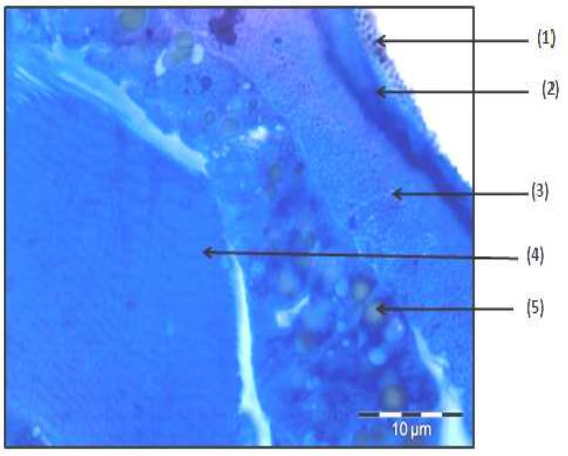
The results for the untreated larvae (control) are shown in Fig (1) which shows that the all layers of the integument are tightly connected together , without any separation , regular and normal morphology of all layers but after 24hrs of treatment with the LD_{25} of lufenuron as illustrated in Fig(2) there are some observations , obvious separation of the hypodermis layer from the endocuticle, abnormalities in the epicuticle morphology , rupture in muscles, irregular and great disintegration of cuticle and hypodermis. These results may explain the sensitivity of treated larvae with Lufenuron to Chlorpyrifos and Imidacloprid.

Table 3. *In-vivo* interaction of tested insecticides with acid and Alkaline phosphatase.

Treatment	Acid phosphatase		Alkaline phosphatase	
	Enzyme activity	% Change of control	Enzyme activity	% Change of control
Control	0.032^{a}	-------	0.009^{c}	-------
Lufenuron	0.01045^{b}	-67.34	0.0124^{b}	+37.3
Imidacloprid	0.01069^{b}	-66.59	0.0125^{b}	+38.3
Chlopyrifos	0.01188^{b}	-62.87	0.0154^{a}	+71.11

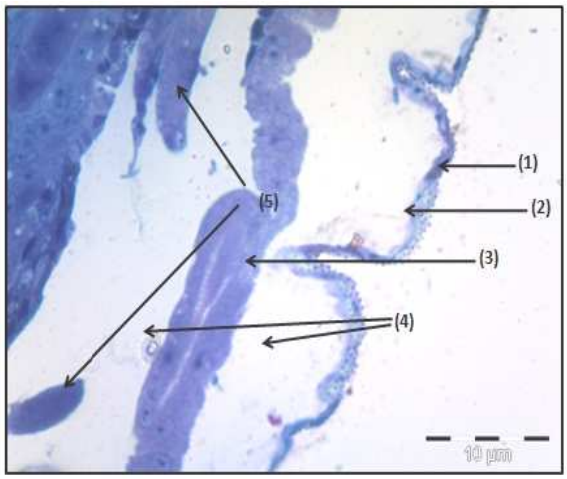
Values followed by the same letters are not significantly different at p <0.05

*** Enzyme activity measured as µmole of substrate hydrolyzed per mg protein per min.**



1-Epicuticle 2-Endocuticle 3- Hybodermis 4- Muscles 5- Fats

Fig. 1a. A photomicrograph of cross section of un-treated larvae of Spodoptera littoralis showing the normal integument layers which appear connected tightly together



1-Epicuticle 2-Endocuticle 3- Hybodermis 4- Degenerated muscles 5- Vacules

Fig. 1b. A photomicrograph of cross section of treated larvae of *Spodoptera littoralis* with LD_{25} of Lufenuron showing the changes in integument layers and muscles.

DISCUSSION

The results for toxicity tests showed that pre-treatment with Lufenuron caused more toxicity of Chlorpyrifos about 55times and about 250times for Imidacloprid compared the toxicity of each compound alone .Similar investigation obtained by Ismail and Morshedy 2009, who tested interaction effect of Dipel-2X with IGRs (Diflubenzuron; Spiromesifen and Pyriproxyfen) , indicated that pretreated of Dipel-2X with IGRs caused more toxicity effect than single treatment. The current findings are consistent with Ghoneim *et al*., 2012, who evaluated the mixtures of organophosphorus (OP) compounds with IGRs for their potential against resistant field populations of *S. littoralis*. The results of co-toxicity factor against 4th instar larvae found that a high synergism occurred when the OP chlorpyrifos mixed with the IGR hexaflumuron or triflumuron, while additive was occurred when mixed with the IGRs chlorfluazuron, chromafenozide or tobufenozite. Similarity, the mixtures of the OP profenofos with IGRs, profenofos with hexaflumuron produced high synergism.

The histological studies for the treated larvae with the LD_{25} of the chitin synthesis inhibitor (Lufenuron) caused obvious separation of the hypodermis layer from the endocuticle, abnormalities in the epicuticle morphology , rupture in muscles, irregular and great disintegration of cuticle and hypodermis, these results are agree with Sabry and Khedr,2014 who tested the effect of the LC_{50} values of chitin synthesis inhibitors (flufenoxuron and teflubenzuron) on the 4th instar larvae of cotton leaf worm *(Spodoptera Littoralis*) then after 48hrs the results showed that Flufenxuron revealed a separation of the hypodermal cells from endocuticle, destruction of basement membrane and appearance of vacuoles between endocuticle and hypodermis. Teflubenzron showed severe damage on the integument, the epidermal cells were highly

impaired being irregular and complete disintegration in the hypodermis was obvious. The two chitin synthesis inhibitors compounds have dystrophic changes on larval muscles where the myofibrils grouped into masses separated by vacuoles and fissure.

Acid Phosphatase (ACP) and Alkaline phosphatase (ALP) are important phosphatases which both differ in their sub cellular distribution. ACP activity is found to be highly concentrated in plasma membrane enriched fraction, where as ALP is associated with lysosomes. They have a very significant role in bivalve immunity. These enzymes are involved in a variety of metabolic activities such as, growth and cell differentiation, protein synthesis, permeability, gonadal maturation, absorption and transport of nutrients, and steroidogenesis (Ram and Sathyanesan, 1985). In this study all treatment led to significant increase in the activity of alkaline phosphatase while caused significant decrease in acid phosphatase.

These results consistent with Reda *et al* (2010) who found that the treated larvae of *Spodoptera littoralis* in both 2nd and 4th larval instars with the sublethal doses LC_{25}, LC_{50} and LC_{90} of flufenoxuron (chitin synthesis inhibitor) showed a significant decrease in enzyme activity of acid phosphatase. In a study by Ewa Jastrzębska (2011), studied the change in soil phosphatase enzymes after application of the insecticides chlorpyrifos and teflubenzuron at different doses and found that teflubenzuron caused increase in alkaline phosphatase activity which agree with our results for Lufenuron. In comparison with teflubenzuron, chlorpyrifos diminished the activity of dehydrogenases, urease, and alkaline phosphatase to a greater while in a study by Pandey and Singh (2006) the use of chlorpyrifos for seed dressing in doses recommended by the manufacturer led to a drop in the activity of alkaline phosphatase. Anindita and Sanjat (2013) investigated the effect of Imidacloprid and Dimethoate on soil phosphatase, the acid phosphatase activity was increased by 24.37% in imidacloprid treated soil and decreased by 23.65% in dimethoate treated soil up to 15 days. Similar trend was also reported for alkaline phosphatase activity where there was an increase of 22.87% in imidacloprid treated soil and diminished by 31.77% in dimethoate treated soil.

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تأثير المعاملة بمركب الليوفينيورون على التغيرات الهسيتولوجية وسمية كلاً من مبيدى الكلوربيريفوس والإيميداكلوبراید ضد يرقات دودة ورق القطن.

نورة عبد الفتاح حسن ، محمود مرشدى و نادر شاكر

كلية الزراعة – جامعة الاسكندرية – الشاطبى

ينتمى كلا من مبيدى الكلوربيريفوس والإيميداكلوبراید إلى مجموعتين رئيستين من المبيدات الحشرية المستخدمة على نطاق واسع فى مكافحة الحشرات , وهناك العديد من الأبحاث التى درست تأثير هاتان المجموعتان على الكائنات غير المستهدفة والبيئة, لذا فمن الضرورى محاولة تقليل الجرعات المستخدمة من هاتان المجموعتان للحفاظ على البيئة .تهدف هذه الدراسة الى تقييم تأثير المعاملة المسبقة بالجرعة غير المميته LD_{25} من مركب الليوفينيورون (مثبطات تخليق الكيتين) على كفاءة كلا من الكلوربيريفوس والإيميداكلوبراید ضد يرقات دودة ورق القطن وذلك بإستخدام طريقة المعاملة السطحية, أوضحت النتائج أن سمية كلا المركبين زادت بعد المعاملة بقيمة LD_{25} من مركب الليوفينيورون بما يقارب حوالى 55 و250 ضعف سمية كلا من الكلوربيريفوس والإيميداكلوبراید على التوالى مقارنة بسمية كل مبيد بمفرده. أوضحت الدراسات الهيستولوجية على يرقات العمر الثانى المعاملة بقيمة LD_{25} من مركب الليوفينيورون حدوث إنفصال واضح لطبقة الهيبودرمس من طبقة الكيوتيكل الداخلى , وتغيرات فى الشكل الظاهرى لطبقة الكيوتيكل السطحى كذلك تمزق للعضلات وعدم إنتظام واضح فى طبقتى الكيوتيكل والهيبودرمس. عند دراسة تأثير كلا من الكلوربيريفوس والإيميداكلوبراید والليوفينيورون In-vivo على نشاط إنزيمى Alkaline phosphatase و Acid phosphatase بإستخدام الجرعة النصف مميتة LD_{50} لكل مركب , أظهرت النتائج حدوث زيادة بفارق معنوى فى نشاط إنزيم Alkaline phosphatase فى المعاملات بينما حدث إنخفاض معنوى لنشاط إنزيم Acid phosphatase أيضا فى كل المعاملات .