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BIOCHEMICAL CHANGES IN A FIELD STRAIN OF *Spodoptera littoralis* (BOISD.) (LEPIDOPTERA: NOCTUIDAE) AFTER EXPOSURE TO THE FIELD RATES OF NEONICOTINOID INSECTICIDES IN ARTIFICIAL DIET

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ABSTRACT

Neonicotinoid insecticides such as thiamethoxam (TMX), thiacloprid (TCD), imidacloprid (ICD) and acetamprid (AMD) are selective nicotinic acetylcholine receptors (nAChRs), and their use has been increasing exponentially. The work aimed to investigate the effects of these compounds on biochemical markers of the 4th larval instar of *Spodoptera littoralis*. TMX, TCD, ICD and AMD were individually mixed in artificial diet at field application rate. The fourth instar larvae were allowed to feed on contaminated or clean diets as untreated control for 1 and 24 hr. The biomarkers of total protein (TP), alanine aminotransferases (ALT) and aspartate aminotransferases (AST), alkaline phosphatase (ALP), antioxidants enzymes (superoxide dismutase (SOD) and glutathione-S-transferase (GST), acetylcholinesterase (AChE) and general esterases were determined in the whole larval homogenates. Results showed that ICD, significantly decreased TP, ALT, AST, ALP and AChE after 24 hr., of exposure. TCD, significantly decreased ALP and SOD (24 hr., of exposure) and GST (after 1 and 24 hr., of exposure). Unexpectedly, TCD induced AChE activity after 24 hr., of exposure. Colorimetric determination of esterases showed significant increase in activity after 1 and 24 hr., of exposure to ICD recording 268.6 and 282.1 nmol/min/mg protein compared with 92.3 and 100.8 nmol/min/mg protein of control, respectively. The obtained results were confirmed electrophoretically by native gel electrophoresis that showed high intensity bands in ICD lane. Also, significant increase in esterase activity was obtained in groups treated with TCD and AMD after 24 hr., of exposure. The current data revealed that the tested neonicotinoid insecticides have different adverse effects on biomarkers of *S. littoralis* that reflects different manners of insect response towards neonicotinoid with the important role of esterases in the tested insecticides detoxification.

Key words: *Spodoptera littoralis*, neonicotinoid insecticides, biochemical markers, AChE, esterases.

INTRODUCTION

The cotton leaf worm, *Spodoptera littoralis* (Boisd.), has a distribution role in regions of northern Africa, Middle East and Mediterranean. It is responsible for the greatest part of losses in several economically important crops such as cotton, corn, peanut, vegetables and soybean (Pineda *et al.*, 2007). Feeding has been observed on at least 84 plant species within 40 families (Brown and Dewhurst, 1975). The chemical control of *S. littoralis* in Egypt has been extensively reported with more than 40 insecticide formulations belonging to different groups of insecticides (El-Sheikh, 2015). The overlapping of crops that serve as hosts of this insect throughout the annual cropping cycle encourages existing high population densities. As a result of high densities and the intensive use of chemical control strategies to manage this pest, development of resistance has resulted to almost all classes of insecticides used (Abo-Elghar *et al.*, 2005).

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Neonicotinoids are among the most effective insecticides for the control of sucking insect pests such as aphids, whiteflies, leaf- and plant hoppers, thrips, some lepidopteran and a number of coleopteran pests (Elbert *et al.*, 2008). This class of insecticides is rapidly expanding since the launch of the first compound imidacloprid (ICD) in 1991 (Elbert and Overbeck, 1990; Elbert *et al.*, 1991) due to its unique characteristics of broad spectrum of efficacy, together with systemic and translaminar action, pronounced residual activity and a unique mode of action. Six additional neonicotinoid insecticides of acetamiprid (AMD), nitenpyram (NPM), thiacloprid (TCD), thiamethoxam (TMX), clothianidin (CTD) and dinotefuran (DNF) were launched in the 10 years after ICD introduction (Minamida *et al.*, 1993; Matsuda and Takahashi, 1996; Kodaka *et al.*, 1998; Elbert *et al.*, 2000; Maienfisch *et al.*, 2001; Altmann and Poncho, 2003). Acting as agonists on nicotinic acetylcholine receptor (nAChR), they are effective in control of pest populations resistant to conventional insecticides. They exhibit long-lasting residual effects as this group shows high systemic and versatile application methods. Combined with high operator and consumer safety, neonicotinoids class products become ideal tools for modern agriculture. Subsequently, sales have nearly doubled in the last few years and expected to get future benefit from organophosphorous (OP) restrictions (Cheung *et al.*, 2006; Elbert *et al.*, 2008).

This study aimed to investigate the biochemical effects of neonicotinoid insecticides on *S. littoralis*. Because neonicotinoids function as agonists of nAChRs, the activity of AChE as a biomarker of synaptic system was determined. Esterase activity in larval homogenates for studying detoxification capability in *S. littoralis* larvae was also determined using colorimetric and electrophoretic methods. Interestingly, ICD showed to inhibit AChE and induce esterases in the 4th instar larvae of *S. littoralis*.

MATERIALS AND METHODS

Insect Rearing

A field strain of the Egyptian cotton leafworm, *Spodoptera littoralis*, was collected from cotton fields of Sharkia Governorate as egg masses and used in the current study. After eggs hatching, larvae were provided with a modified version of artificial diet for insect rearing in the laboratory at 27±2 ºC, 60 - 70% relative humidity and 16 : 8 hr., (light : dark) photoperiod. The diet was prepared according to the method mentioned in El-Sheikh (2015). Briefly, 220 g of white beans, 14 g of powdered agar, 4.8 g of ascorbic acid, 0.9 g of sorbic acid, 2.4 g of methyl-*p*-hydroxybenzoate, 40 g of brewer's yeast and 700 ml of distilled water were used for preparing 1 L of diet. Next generation was used in the study, which the required larvae for experiments were obtained by placing 50 - 60 pupae in wide glass gars until adult emergence. The emerged adults were provided with clean branches of tafla (*Nerium oleander*) for oviposition and supplied with 10% (*W/V*) of bee honey solution. Tafla leaves with freshly deposited eggs were collected daily and placed in a container (12 × 18 × 5 cm) provided with artificial diet for insect rearing.

Insecticides and Chemicals Used

Neonicotinoid insecticides of thiamethoxam (TMX; Thiamex 25% WG), thiacloprid (TCD; Blanch 48% SC), imidacloprid (ICD; Imidazed 20% SC) and acetamiprid (AMD; Mospilan 20% SG) (Fig. 1) were used in the current study. Kits of total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), were purchased from Al-Gomhoria Company for Medical Needs and Laboratory Tools. Kit of alkaline phosphatase (ALP) was obtained from Hi-Lab., Zagazig, Egypt. Superoxide dismutase (SOD), glutathione-S-transferase (GST) and acetylcholinesterase (AChE) were obtained from Bio-diagnostic Company, Cairo, Egypt. Fast Blue B salt was obtained from Sigma-Aldrich (St. Louis, MO, USA) and α-naphthyl actetate (α-NA) was purchased from Alfa Aesar Company (Karlsruhe, Germany). All used commercial reagents and other chemicals were of technical grade with the highest purity available.

Treatments

Field application rates of TMX, TCD, ICD or AMD (200, 576, 250 or 50 µg a.i. ml^{-1} diet, respectively) well mixed with semi-synthetic diet. Contaminated diets were prepared by mixing each insecticide-concentration individually with the previously mentioned diet at ~35 ºC.

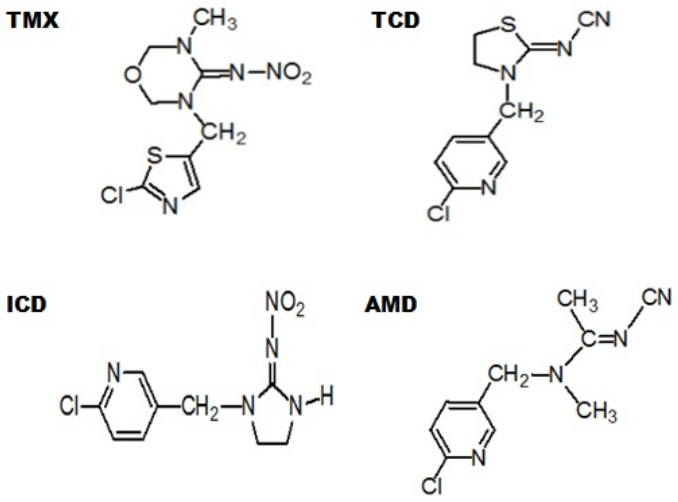

Fig. 1. Insecticides used in the study: thiamethoxam (TMX), thiacloprid (TCD), imidacloprid (ICD) and acetamiprid (AMD)

Ten 4th instar larvae were transferred into 9 cm glass petri-dishes contain cubes of clean diet (control) or each contaminated diet (treatments). Six replicates of each insecticide were used, so the total number of 60 larvae/ insecticide were used. After 1 hr., and 24 hr., of exposure, 5 alive larvae/replicate were randomly transfered into 1.5 ml micro-centrifuge tube in three replicates for each time, so the total larvae of 15 were used. Collected larvae were frozen directly for 24 hr., at -20 ºC, and then homogenized.

Preparation of Larval Homogenates

Five larvae per replicate were homogenized in ice-cooled 0.1 M phosphate buffer (pH 7.4) containing 0.02% Triton X-100 and 0.05% 1-phenyl-2-thiourea (PTU) to prevent melanization of the homogenates in a rate of (100 mg/200 µl, *W/V*) using Universal Laboratory Aid homogenizer, Type MPW-309 (Mechanika Precyzyjna, Warsaw, Poland) on ice at 1000 rpm for 60 sec. Larval homogenates were centrifuged at 15000 rpm for 10 minutes at 4 ºC. Supernatants were filtered using filter paper of Whatmann 1, and then kept at -20 ºC until determination within 2 weeks.

Biochemical Determinations

TP was determined in the larval homogenates after 1 hr., and 24 hr., of exposure to diet-containing with field rates of neonicotinoid insecticides using the method of Lowry *et al.* (1951). ALT and AST activities were determined according to the method of Reitman and Frankel (1957). ALP activity was determined according to the method of Kind and King (1954). The resulting supernatants of larval homogenates were used in measuring activities of SOD, GST and AChE according to methods described by Kakkar *et al.* (1984), Grant and Matsumura (1988) and Ellman *et al.* (1961), respectively.

Esterase Determination by Colorimetric and Electrophoresis Methods

Colorimetric esterase activity determination was done using the general substrates α-NA as described by Gomori (1953) with modification. A 300 µl total volume of reaction mixtures/well is consisting of 80 µl 0.1 M phosphate buffer (pH 7.4) containing 0.02% Triton X-100, 20 µl of larval homogenate, 100 µl of α-NA substrate solution (final concentration = 2.5 mM) and 100 µl of Fast Blue B salt solution (consisting of 30 µl of 1% Fast Blue B salt + 70 µl of 5% SDS). Each homogenate sample for each treatment or control was replicated 3 times and incubated at 30 ºC for 5 min before measurement in 96-well microplates using microplate autoreader, Bio-TEK Instrument, Highland Park, Winooski, VT, according to Ashour *et al*. (1987). After incubation, the optical density (OD) was measured at 450 nm and the activity of esterase was expressed as nmol min^{-1} mg^{-1} protein.

Nondenaturing polyacrylamide gel electrophoresis (Native PAGE) was performed using 7.5% native polyacrylamide gels according to

the method of Davis (1964). Esterase bands were visualized by incubation of gels with 0.04% (*W/V*) α-NA, 0.1% (*W/V*) Fast Blue B salt in 100 mM phosphate buffer, pH 7.4.

Statistical Analysis

The differences in biochemical measurements were performed between treatments and control. SPSS 14.0 for Windows software package was used through least significant difference (LSD) of One-Way ANOVA at $p<0.05$ to statistically analyze the data.

RESULTS AND DISCUSSION

Concentrations of TP (Fig. 2) were insignificantly decreased in the larvae treated by TCD and ICD for 1 hr. This decrease was continued for 24 hr., which was significant ($p<0.05$) in ICD-treated larvae, when compared with control.

The effect of neonicotinoid insecticides on transaminase enzymes of AST and ALT (Figs. 3 and 4, respectively) showed that both enzymes were insignificantly decreased due to exposure to TCD and ICD for 1 hr., while their activity significantly ($p<0.05$) decreased after 24 hr., of exposure in ICD-treated larvae comparing with control.

ALP activity (Fig. 5) showed normal as compared with control after 1 hr., of exposure to neonicotinoid insecticides, while significant ($p<0.05$) decrease was observed in the activity of ALP in larval homogenates of groups treated with TCD or ICD.

Aminotransferases are important enzymes in the synthesis of amino acids, which form proteins. In hemolymph of *Eurygaster integriceps*, the activity levels of ALT, AST and ALP increased while TP was decreased due to treatment with pyriproxyfen. These changes have been supported by several studies due to using different insecticides (Zibaee *et al.*, 2011). Insect ALP is distributed in different insect organs and tissues. They might be involved in the different vital processes in insects and can be targeted by insecticides (Ying *et al.*, 2009). Both TCD and ICD, significantly inhibited ALP activity after 24 hr., of exposure suggesting that these insecticides have adverse effects on biological processes in *S. littoralis*.

The effect of the tested insecticides on the activity of antioxidant enzymes are shown in Table 1. When larvae of *S. littoralis* exposed to the diet incorporated with a field rate of each neonicotinoid insecticide for 1 hr., or 24 hr., SOD and GST were significantly ($p<0.05$) decreased due to exposure to TCD comparing the same enzymes in control larvae. The rest treatments showed insignificant change when compared with control measurements. Glutathione-S-transferases (GSTs) play a pivotal role in detoxifying endogenous and xenobiotic compounds and have a role of oxidative stress resistance in cells (Grant *et al*., 1989; Huang *et al.*, 2011). SOD has an important role as antioxidant defense in nearly all living cells exposed to oxygen (El-Sheikh and Galal, 2015) which could lead to generation of reactive oxygen species (ROS). GSTs from some insects possess selenium-independent peroxidase activity and metabolize hydroperoxides (*i.e.*, 4-hydroxynonenal and malonaldehyde) by catalyzing the conjugation of these lipid peroxidation end products with GSH (Singh *et al.*, 2001; Krishnan and Kodrik, 2006). Not only hydroperoxides, but also some bacteria can induce oxidative stress in the insect midgut, resulting in the increased activity of some antioxidative enzymes such as GSTs (Dubovskiy *et al.*, 2008). In the current study, SOD and GST was determined as antioxidant enzymes which significantly decreased due to exposure to TCD suggesting that this insecticide might induce oxidative stress in *S. littoralis*.

AChE activity (Table 2) did not change significantly after 1 hr., of exposure to the tested insecticides even its activity showed higher than control in TCD-treated insects. After 24 hr., of exposure, AChE activity showed significant ($p<0.05$) increase in TCD-treated larvae, while showed significant decrease in ICD-treated larvae when compared with control. Neonicotinoids are agonist of nAChRs (Tan *et al.*, 2007) and they do not show a direct inhibition in activity of AChE comparing to a known inhibitor group of organophosphate

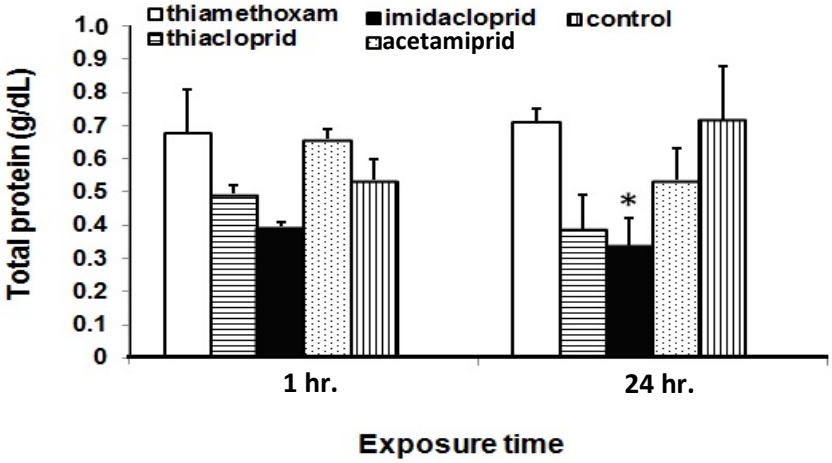

Fig. 2. Total protein (TP) concentration in the 4th larval instar homogenates of *S. littoralis* after 1 hr., and 24 hr., of feeding on artificial diet incorporated with field rates of the tested neonicotinoid insecticides

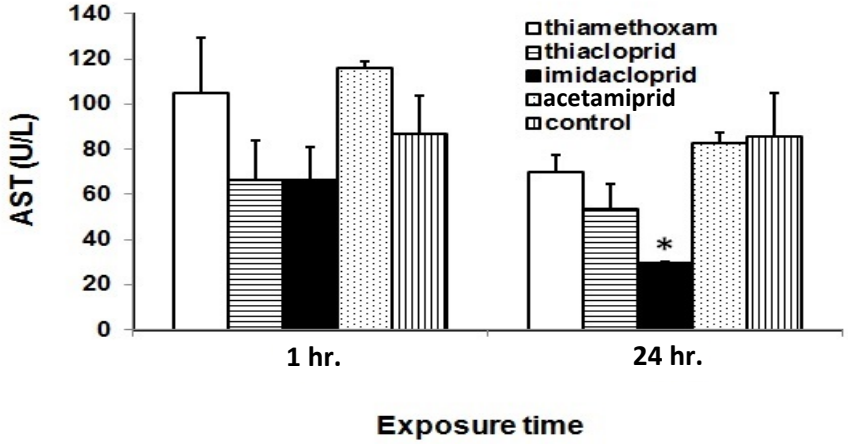

Fig. 3. Aspartate aminotransferase (AST) activity in the 4th larval instar homogenates of *S. littoralis* after 1 hr., and 24 hr., of feeding on artificial diet incorporated with field rates of the tested neonicotinoid insecticides

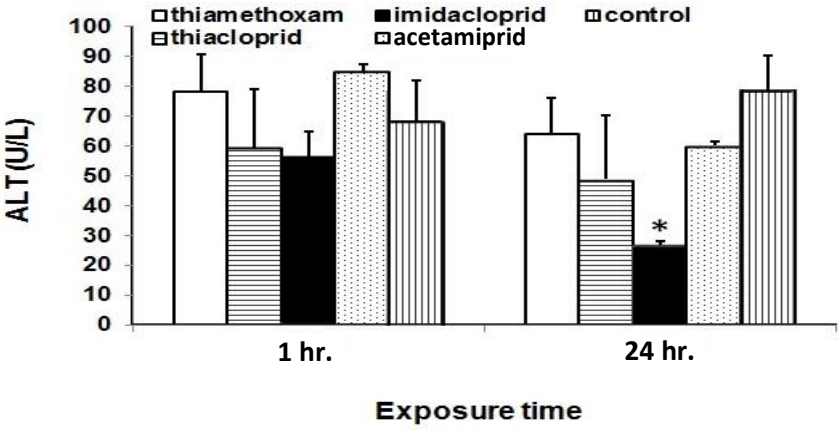

Fig. 4. Alanine aminotransferase (ALT) activity in the 4th larval instar homogenates of *S. littoralis* after 1 hr., and 24 hr., of feeding on artificial diet incorporated with field rates of the tested neonicotinoid insecticides

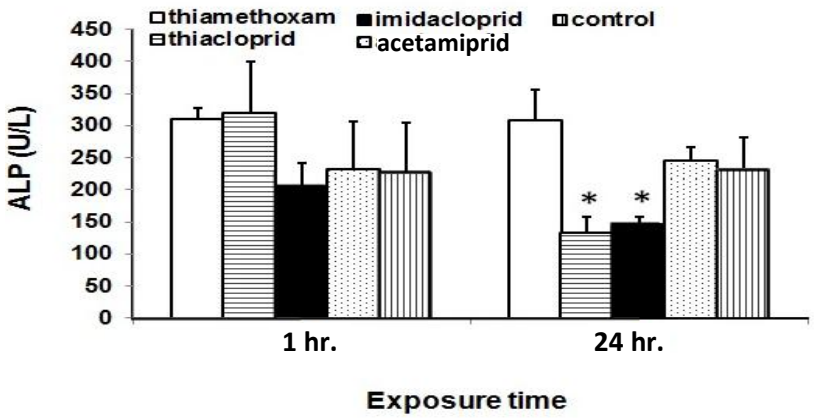

Fig. 5. Alkaline phosphatase (ALP) activity in the 4th larval instar homogenates of *S. littorals* after 1 hr., and 24 hr., of feeding on artificial diet incorporated with field rates of the tested neonicotinoid insecticides

Table 1. Superoxide dismutase (SOD) and Glutathione S- transferase (GST) activities in the 4th larval instar homogenates of *S. littoralis* after 1 hr., and 24 hr., of feeding on artificial diet incorporated with field rates of the tested neonicotinoid insecticides

Insecticide	SOD (U/L)		GST (ng/ml)	
	1 hr.	24 hr.	1 hr.	24 hr.
Thiamethoxam	11.6±1.9	20.7±1.9	0.42±0.02	0.61±0.01
Thiacloprid	5.1±2.3*	4.3±1.2*	0.12±0.05*	0.15±0.04*
Imidacloprid	25.6±9.3	22.3±6.3	0.49±0.05	0.61±0.07
Acetamiprid	34.2±13.7	11.4±3.9	0.44±0.21	0.52±0.18
Control	19.9±5.5	18.0±4.9	0.45±0.03	0.42±0.03

Table 2. Acetylcholinesterase (AChE) activity in the 4th larval instar homogenates of *S. littoralis* after 1 hr., and 24 hr., of feeding on artificial diet incorporated with field rates of the tested neonicotinoid insecticides

Insecticide	AChE (U/L)		Ratio (treated/control)	
	1 hr.	24 hr.	1 hr.	24 hr.
Thiamethoxam	179.6±1.3	157.4±35.7	1.4	0.9
Thiacloprid	248.3±11.2*	264.8±27.6*	1.9	1.6
Imidacloprid	147.9±57.3	74.5±21.5*	1.1	0.4
Acetamiprid	154.4±48.0	197.6±17.6	1.2	1.2
Control	131.7±14.4	169.6±12.2	1.0	1.0

insecticides (Dondero *et al.*, 2010). However, we tested such effects on *S. littoralis* after exposure to neonicotinoid insecticides as a functional parameter of the cholinergic system. We found two patterns of response to the tested insecticides, a significant ($p<0.05$) increase in the activity of AChE in TCD-treated larvae after 24hr., of exposure and a significant decrease in ICD-treated larvae as compared with control. Although there are few data in literature on the effects of neonicotinoids on AChE activity, the current findings agree with what reported in the study of Dondero *et al.* (2010) as they showed that TCD significantly increased AChE activity in marine mussels after exposure to a concentration of 1 mg/l, while ICD significantly inhibited enzyme activity after exposure to the same concentration. German cockroaches AChE activity could be modulated by AMD insecticide (Morakchi *et al.*, 2005), while other species such as *Daphnia magna* (Tišler *et al.*, 2009) and the earthworm, *Aporrectodea nocturna*, (Capowiez *et al.*, 2003) exposed to ICD displayed no effects. Depending on previous reports, AChE responses seem to depend on species and probably individual neonicotinoid insecticides.

The data of general esterases presented in Table 3 show increase in total homogenate activity after exposure to neonicotinoid insecticides for 1 or 24 hr. Increase levels of esterases were significant ($p<0.05$) in ICD-treated larvae after 1 hr., which showed 2.9 folds higher than control larvae. After 24 hr., of exposure, esterase activity increased significantly ($p<0.05$) in larvae treated with TCD, ICD and AMD which amounted 2.0, 2.8 and 2.2 folds higher than control, respectively. The current data are consistent with what Buès *et al.* (2005) obtained, as they found that esterase activity was 2.6- and 3.0-times higher in deltamethrin-resistant and methomyl-resistant strains of *Helicoverpa armigera* compared with its field strain. The current data show that larval exposure to ICD greatly induced esterases even after short time of exposure (1 hr.), which could be a reason for low effectiveness of ICD compared with other insecticides. The obtained results of colorimetric determination of esterases were confirmed in native gel electrophoresis (Fig. 6). Non-denaturing polyacrylamide gel electrophoresis showed esterase band patterns different in size, weight and density among neonicotinoid insecticides and control. Lane of ICD-treated larvae showed high intensity of esterase isozymes comparing with other insecticides and control. The differences in esterase activity may be associated with individual isozyme activity or the genetic variations among strains as showed in *S. litura* selected with deltamethrin and chlorpyrifos-methyl (Cho *et al.*, 1999) that displayed high levels of non specific esterase activity and more intensified esterase bands (Ono *et al.*, 1994).

In conclusion, neonicotinoid insecticide of ICD showed to affect biochemical markers in *S. littoralis*, followed by TCD. Excepting TMX, other tested insecticides showed to significantly induce esterase activity that could be a reason for detoxification and a high toxic effect of TMX on the tested insect.

Table 3. General esterase activity in the 4th larval instar homogenates of *S. littoralis* after 1 hr., and 24 hr., of feeding on artificial diet incorporated with field rates of the tested neonicotinoid insecticides

Insecticide	Esterase activity (nmol/min/mg protein)		Ratio (treated/control)	
	1 hr.	24 hr.	1 hr.	24 hr.
Thiamethoxam	142.5±5.9	129.5±18.2	1.5	1.3
Thiacloprid	126.4±6.9	203.4±23.0*	1.5	2.0
Imidacloprid	268.6±50.4*	282.1±35.7*	2.9	2.8
Acetamiprid	172.3±27.7	222.6±44.7*	1.9	2.2
Control	92.3±14.9	100.8±13.0	1.0	1.0

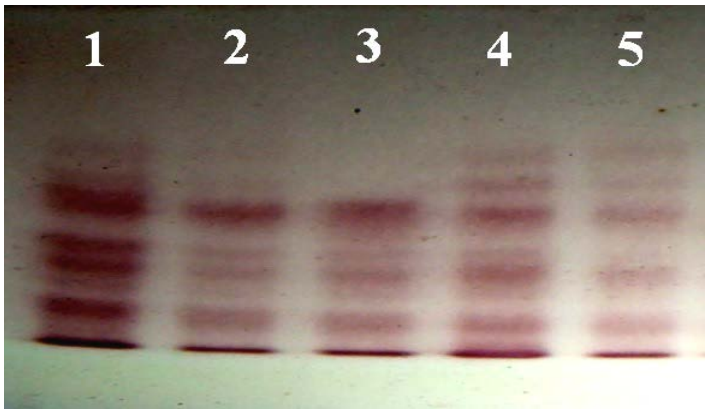

Fig. 6. Native gel electrophoresis of 7.5% PAGE run for 1 hr., on constant current (90 mA) and for 3 hr., on 120 mA for samples of *S. littoralis* larval homogenates exposed for 24 hr., to field rates of neonicotinoids in diet. A volume of 20 µl of each sample was loaded in each well. Lanes from 1-5 are representing larval homogenates of groups exposed to imidacloprid, acetamiprid, thiamethoxam, thiacloprid and control, respectively. Gel stained for 60 min at 35ºC with 100 mg fast blue B salt and 40 mg α-NA in 100 ml phosphate buffer (0.1 M, pH 7.4)

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التغيرات البيوكيميائية في سلالة حقلية لدودة ورق القطن (*Spodoptera littoralis* (Biosd.
بعد التعرض للمعدلات الحقلية من مبيدات النيونيكوتين الحشرية في البيئة الصناعية

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المبيدات الحشرية التابعة لمجموعة النيونيكوتين مثل الثياميثوكسام، الثياكلوبريد، الإيميداكلوبريد والأستيامبريد تعمل بشكل اختياري علي مستقبلات الأسيتايل كولين النيكوتينية، ويزداد إستخدامها بشكل ملحوظ، يهدف هذا العمل إلي دراسة تأثيرات هذه المبيدات على الدلائل البيوكيميائية في يرقات العمر الرابع لدودة ورق القطن، لإنجاز هذا الهدف، تم خلط المعدل الحقلي من كل مبيد بشكل منفرد مع البيئة الصناعية بمعدل مساوي للمعدل الحقلي، تُركت يرقات العمر الرابع للتغذية على البيئة المخلوطة بالمبيدات أو البيئة النظيفة (كنترول) لمدة ساعة ولمدة ٢٤ ساعة، تم تقدير الدلائل الخاصة بالبروتين الكلي، الإنزيمات الناقلة للأمين (AST, ALT)، الألكالين فوسفاتيز(ALP)، الإنزيمات المضادة للأكسدة (SOD, GST)، الأسيتايل كولين (AChE) والإستيريزس العامة، وذلك في مطحون اليرقات، أوضحت النتائج أن المعاملة بمبيد الإيميداكلوبريد تسببت في نقص معنوي بكل من TP، ALT، AST، ALP و AChE بعد ٢٤ ساعة من المعاملة، أظهر مبيد الثياكلوبريد نقص معنوي في كل من ALP وSOD (بعد ٢٤ ساعة من التعرض) و GST (بعد ساعة وبعد ٢٤ ساعة من التعرض)، أظهر مبيد الثياكلوبريد، بشكل غير متوقع، تحفيز لنشاط AChE بعد ٢٤ ساعة من التعرض، أظهر التقدير اللوني للإستيريزس زيادة معنوية في النشاط بعد ساعة وبعد ٢٤ ساعة من التعرض لمبيد الإيميداكلوبريد حيث سجل ٢٦٨.٦ و ٢٨٢.١ نانومول / دقيقة / مليجرام بروتين مقارنة بـ ٩٢,٣ و ١٠٠.٨ نانومول / دقيقة/ مليجرام بروتين في الكنترول، علي الترتيب، تم تأكيد النتائج المتحصل عليها بالتفريد الكهربي الذي أظهر حِزَم عالية الكثافة في المجموعة المعاملة بالإيميداكلوبريد، كما تم الحصول على زيادة معنوية في نشاط الإستيريزس في اليرقات التي تعرضت لكل من الثياكلوبريد والأسيتامبريد بعد ٢٤ ساعة من التعرض، أظهرت النتائج أن المبيدات المختبرة لها تأثيرات مختلفة علي الدلائل الحيوية في دودة ورق القطن والتي تعكس أساليب استجابة الحشرة تجاه المبيدات المختبرة مع أهمية دور الإستيريزس في إزالة سميتها.

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