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Changes in detoxifying enzymes and carbohydrate metabolism associated with spinetoram in two field-collected strains of *Spodoptera littoralis* (Biosd.)

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ABSTRACT

This study was conducted to investigate the biochemical defensive mechanisms in larvae of *S. littoralis* (Biosd.) (Lepidoptera: Noctuidae) collected from cotton fields in two Egyptian Governorates (Kalyobia and Behira), sprayed with spinetoram as well as its impact effect upon carbohydrate metabolism. The role played of spinetoram on different major defensive enzymes like glutathione S-transferases (GSTs) , non specific esterases and acetylcholinesterase(AChE) was discussed. It seems that these enzymes are not greatly involved in the detoxifying process of spinetoram except **a** marked over production of AChE reached up to 18.7% as well as the concentration of its substrate, ACh with an increase reached up to 42.8%. Spinetoram had also a prominent effect upon carbohydrate metabolism as in glycogen content which was decreased by 34.9%, LDH also was inhibited to 55.7% while trehalase had an elevated activity by 22.8%.In general, Behira Governorate was more affected and more sensitive to spinetoram rather than Kalyobia Governorate. The present work is an introductory study to understand the outline of the mechanism of this bioinsecticide as well as the resistance mechanisms may arise in the future.

INTRODUCTION

The cotton leaf worm, *Spodoptera littoralis* (Biosd.) (Lepidoptera: Noctuidae), is a serious polyphagous pest that damages numerous kinds of cultivated crops, including corn, cotton, beet, tomato, and many others .Due to overuse of insecticides over the past years, *S. littoralis* has developed resistance to various synthetic insecticides.

Bioinsecticides are currently studied more and more because of the possibility of their use in plant protection as an alternative method to the broad use of conventional pesticides. Nowadays, there are several novel insecticides which show good activities against the cotton leaf worm.

Spinetoram is one of these bioinsecticides. It is a mixture of spinosyns A and D, and derived from the naturally occurring soil actinomycete *Saccharopolyspora spinosa* (Sparks *et al.*, 1998). Because of its unique action mechanism, as it has strong insecticidal activity especially against lepidoptera larvae with low levels of mammalian toxicity and relatively little toxicity to nontarget insects (Bret *et al.*,1997). However, any insecticide can develop resistance in target insects from the insight of organic evolution. Recently, several insects have exhibited a rapid threatening ascending resistance to Spinetoram in field populations in recent years. (John *et al.*, 2000 and Zhao *et al.*, 2002). However, its precise mode of action on insects has not been well established till now and several hypotheses have been proposed, as it appears to be of a unique mechanism, with a primary site of attack being the nicotinic acetylcholine receptor and a secondary site of attack being GABA receptors (Watson, 2001). Routes of entry include contact and oral and it causes involuntary muscular contractions. Because of the prolonged hyperexcitation, insects eventually became paralyzed, apparently due to neuromuscular fatigue (Salgado *et al.*, 1998).

Several defensive mechanisms and biochemical reactions are involved in the detoxification processes against any chemical intruders, i.e. insecticides. These mechanisms predominantly involve either metabolic detoxification of the insecticide before it reaches its target site, or the sensitivity changes of the target site so that it is no longer susceptible to insecticide inhibition The most common metabolic resistance mechanisms involve esterases, glutathione S-transferases (GSTs). In

most, but not all instances of metabolic resistance, resistant insects can be detected through increased quantities of such enzymes compared to their susceptible counterparts (Brown and Brogdon, 1987; Hemingway, 1989 and Hemingway *et al.*, 1995).

Accordingly, (GSTs) have attracted attention in insects because of their involvement in the defense towards insecticides mainly organophosphates, organochlorines and cyclodienes (Reidy *et al.*, 1990; Clark *et al.*, 1986; Grant and Matsumura, 1989 and Fournier *et al.*,1992). Reports correlating high levels of GSTs with high resistance to pyrethroids do exist for *S. littoralis* (Lagadic *et al.*,1993) and *Tribolium castaneum* (Reidy *et al.*,1990). Induction of GST by pyrethroids has also been reported for the honey bee (Yu *et al.*, 1984), *S. frugiperda* (Punzo, 1993) and German cockroach (Hemingway *et al.*,1993).

On the other hand, General esterases are a large and diverse group of hydrolases that hydrolyze numerous substrates including esters and certain non-ester compounds. Numerous studies have demonstrated that esterases play an important role in conferring or contributing to insecticide detoxifications in insect and other arthropod species (Mouches *et al.*, 1986).

Acetylcholinesterase (AChE) is a key enzyme in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. In insects, AChE is the only cholinesterase (Salgado, 1998) and possesses a substrate specificity that is intermediate between that of vertebrate acetylcholinesterases. It is one of the most known defensive esterases as it is the major target for organophosphate and carbamate insecticides.

The biochemical responses of some Lepidopterous insects on exposure to different insecticides on certain aspects of carbohydrate metabolism have been well documented (Bhosale and Kallapur, 1985 and Nath *et al.*, 2000). However, information on the effects of these insecticides on glycogen metabolism focusing on lactate dehydrogenase (LDH) and trehalase are still inadequate. LDH is an important glycolytic enzyme involved in carbohydrate metabolism and has been used as an indicative criterion of exposure to chemical stress (Wu and Lam, 1997; Diamantino *et al.*, 2001). On the other hand, trehalase is an important enzyme in which insects degrad trehalose to glucose for internal energy supply (Wyatt, 1967), thus the activity of terhalose might serve as an indicator of energy reserves resulting from availability of carbohydrate nutrients.

Accordingly, the present study is an attempt to (1) offer baseline data of relative contribution of famous detoxifying enzymes. (2) Also, a trial has been made to assess the Spinetoram toxic impact on carbohydrate metabolism in two field strains of *S. littoralis*. This preliminary study will help in future studies concerned to explore strategies for resistance management and prolong the useful life of Spinetoram.

MATERIALS AND METHODS

1. Field Experiments:

The experiments were conducted at Kaha research station, Toukh district, Qalyobia Governorate and Abu Madawey farm at Kafr eldwar district, Behira Governorate to apply (spray) the novel biocide, Radiant (Sc 12%) against cotton leaf worm, *S. littoralis* (Boisd). The field areas were cultivated with Giza86 cotton variety on March, 2008 and the normal agricultural practices were applied. The experimental area in each governorate was divided into plates of 1/16 feddan (262.5 m^2). The treatment was arranged in randomized complete blocks design (RCBD) with four replicates each. Application of insecticide was on July. A motor sprayer was used. The volume of spray solution was 40 liters/feddan.

Tested Compound:-

Spinetoram**.**

Trade name:

Radiant (12 % SC).

Chemical name:

This compound is a mixture of major and minor components:

Major component (3'-ethoxy-5, 6-dihyro spinosyn J).

Minor component (3'-ethoxy spinosyn L).

Biochemical studies:

Sample preparation:

Fifth larval instar of *S. littoralis* larvae were collected to be tested before and on the 7th or 8th day post treatment with previously mentioned recommended dose. The whole larval bodies were homogenized (1gm of tissue in 1 ml of distilled water), using hand glass homogenizer on ice jacket then centrifuged using Eppendorf refrigerated 5415(Hamburg, Germany) at 8000 rpm for 15 at 2°C. The supernatants were kept at -20ºc till use.

Biochemical tests:

1:Glutathion-S-transeferase activity was determined according to the method of Habig *et al.* (1974).

2: Determination of general esterease activities (ά and β-esterases activities) were evaluated according to the method of Van Asperen (1962).

3: The activity of acetyl cholinesterase enzyme and acetylcholine concenterations was determined by the method adopted by Ellman *et al*. (1961) .

4: Lactate dehydrogenase activity was determined according to King (1965).

5: Trehalose hydrolyzing enzyme, trehalase was evaluated according to the method described by Ishaaya & Swirski (1976).

6: Glycogen content was determined according to Dubois (1956).

Statistical analysis:

Data were subjected to statistical analysis using analysis of variance two ways ANOVA (Snedecor & Cochran, 1967) and the least significant difference (LSD) test was used for mean separation at $P \leq 0.05$.

RESULTS

The present data show a significant decrease of GST in the 5th larval instar homogenate after being sprayed in the field with Spinetoram. In Kalyobia and Behira Governorates (Fig. 1), this enzyme decreased by 14.28% and 18.13% compared to the unsprayed ones, respectively.

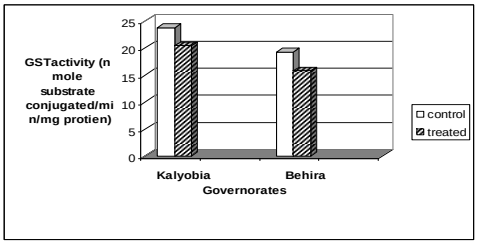

Fig. (1): GST activity in the larval homogenates of the 5th larval instar of *S. littoralis* before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

On the other hand, α and β esterases showed variable values in the two studied strains, in kalyobia governorate, α esterases significantly increased by 1.37 folds in the sprayed larvae relative to the control ones. While an opposite trend was found in Behira Governorate, whereas a highly significant suppression of α esterases reached to 44.65% compared to unsprayed larvae (Fig.2).

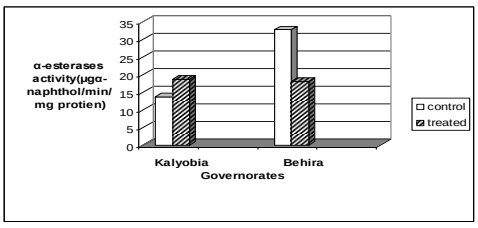

Fig. (2): άesterases activity in the larval homogenates of the 5th larval instar of *S. littoralis* before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

In tracing β esterases, no significant difference was recorded in Kalyobia while there was a highly significant decrease in Behiara Governorate reached 46.58% compared to the control (Fig.3).

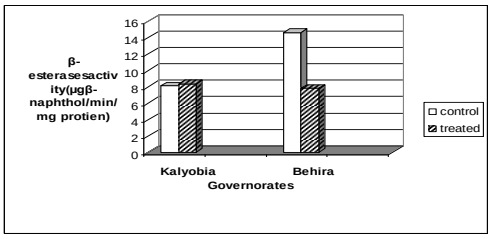

Fig. (3): *β*-esterases activity in the larval homogenates of the 5th larval instar of *S.*

littoralis before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

A significant increase in AChE was detected in the present work by 4.94% compared to the control unsprayed larvae in kalyobia governorate while in Behiara restrict, this significant increase reached 18.65 % as it increased from 271.7±2.9 in the control to 334±7.5 gm AchBr × 10^3/ min /mg protein in the treated larvae (Fig.4).

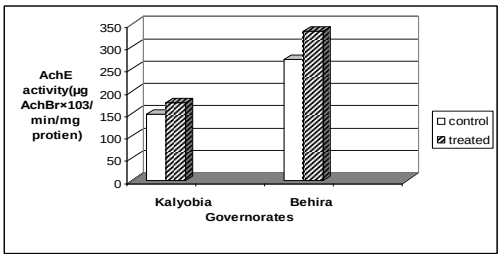

Fig. (4): AChE activity in the larval homogenates of the 5th larval instar of *S. littoralis* before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

Similar trend was achieved in acetylcholine ACh concenteration as it was found an enhancement in production reached 37.35% and 42.8 % compared to control in its Kalyobia and Behiara Governorates, respectively (Fig.5).

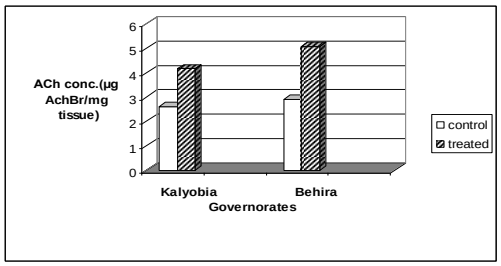

Fig. (5): ACh concentration in the larval homogenates of the 5th larval instar of *S. littoralis* before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

Dealing with carbohydrate metabolism and its correlation with Spinetoram treatment , our data showed an inhibitory effect in LDH as it decreases from 54.3±0.8 in the homogenate of the unsprayed larvae to 29±0.85 U×10^3/mg protein in the treated one being decreased dramatically by 46.59% compared to the control in fields of Kalyobia governorate while in Behiara governorate, the enzyme's level decreased from 60.3±1.1 in the control larvae to 26.7±0.75 U×10^3/mg protein after spraying Spinetoram with nearly 55.75% suppression ratio relative to the control (Fig.6).

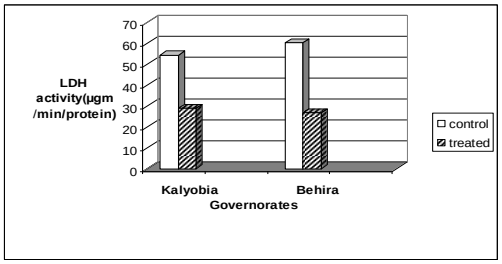

Fig. (6): LDH activity in the larval homogenates of the 5th larval instar of *S. littoralis* before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

Still with carbohydrate metabolism, the present data also shows a significant increase in the production of trehalase, this increase reached 7.72% in our first governorate, Kalyobia while in Behira Governorate; enzyme's activity decreased by 22.83% relative to the control unsprayed larvae (Fig.7).

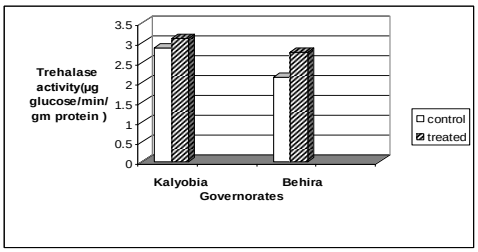

Fig. (7): Trehalase activity in the larval homogenates of the 5th larval instar of *S. littoralis* before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

Glycogen in the present study shows a significant decrease in both governorates, it decreased by 21.9 and 34.91% in Kalyobia and Behiara governorates, respectively (Fig.8).

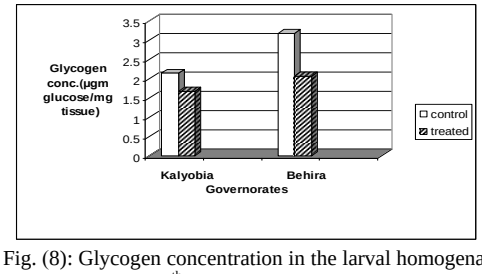

Fig. (8): Glycogen concentration in the larval homogenates of the 5th larval instar of *S. littoralis* before and after being sprayed with Spinetoram.

*Each column depict mean of value recorded in three separate replicates.

DISCUSSION

Generally speaking, increase of activity of detoxification enzymes is the most universal resistant mechanism in insects. Accordingly, we expected in the present work to find an elevation in the activity of such enzymes but surprisingly, this assumption couldn't be achieved as GST relatively decreased after treatment.

The GST system is known to be involved in the metabolization of various endogenous compounds, but is also recognized as one of the major mechanism conferring insecticide resistance in many pests (Yu, 2004). GSTs also play an important role in stress physiology, and have been implicated in intracellular transport and various biosynthetic pathways (Wilce and Parker, 1994).

Esterase-based resistance to organophosphorus and carbamate insecticides is common in a range of different insect pests (Field *et al.,* 1988 and Hemingway and Karunaratne, 1998). The esterases either produce broad spectrum insecticide resistance through rapid-binding and slow turnover of insecticide, i.e. sequestration, or narrow spectrum resistance through metabolism of a very restricted range of insecticides containing a common ester bond (Herath *et al.,* 1987). The majority of esterases which function by sequestration are elevated through gene amplification, (Vaughan and Hemingway, 1995).Since enhanced metabolism is an important insecticide mechanism, thus oxidative, hydrolytic and conjugative detoxication enzyme activities toward universal substrates were measured in insecticide (Abo Elghar *et al.*,2005).

No fixed trend was observed in the activity of general estresases in the present work suggesting that they are not involved in the contribution of the detoxification mechanism. The variable response in the general esterase in the present study, may be due to geographical distribution and subsequently environmental conditions in each tested governorate or my be due to specific characteristics for each strain. On the other hand, we have to mention that an increase in activity of just one of the numerous different enzymes within each enzyme family might be missed by these relatively crude assays.

The general decrease in the activity of the studied enzymes in the present work may indicate that GST and general esterases are not involved in the detoxification process of Spinetoram. These finding go parallel with Wang *et al.* (2009) who found that esterases and GST might be unimportant or less important in conferring spinosad resistance in the *S. exigua* field population. They suggested also that the biochemical mechanisms of insect resistance to spinosad might be related with the species of insect pests. Shono and Jefrey (2003) suggested that the mechanism of resistance to spinosad was not due to metabolic detoxication by monooxygenases, hydrolases, or GST but because of altering of target site. Similarly, Zhang *et al.* (2003) reported that there was no obvious relationship between the sensitivity of the beet armyworm to Spinosad and the activities of endogenous enzymes of protective system.

An exception of our finding was detected during the evaluation of AChE, as the present study shows a marked enhancement in the production of this enzyme as well as its substrate, ACh after being treated with Spinetoram. AChE has a key role in neurotransmission by hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses of the nervous system and is the target site of several neurotoxic insecticides This hyperactivity of AChE and the overproduction of ACh which is found in this study may be explained according to Salgado *et al.* (1998) who has demonstrated that spinosad could attack the nicotinic acetylcholine receptor (nAChR) with acetylcholine ACh simultaneously, as well as acting on a new site differing from the site on which ACh acts. He gave a hypothesis that there were two special sites on nAChR for Spinetoram and ACh individually. When both Spinetoram and ACh are absent, the receptor channel will keep closed. When either of them is present or both of them are present, the channel will open up and subsequently the receptor will be

activated. This assumption may be able to explain the overproduction of both AChE and ACh. However, there is no evidence to demonstrate that spinosad directly links to a site on nAChR, and it probably means that Spinetoram indirectly regulates the nAChR. Furthermore, Watson (2001) indicated that Spinetoram could also act on γ-aminobutyric acid (GABA) receptor and increase neural activity of pest in excess and subsequently make the pest fall into a decline and be dead eventually.

Concerning carbohydrates metabolism, the suppression of (LDH) level due to Spinetoram treatment demonstrating low nutritional efficiency of the larvae which will affect simultaneously all subsequent vital activities since LDH is an important glycolytic enzyme and has been used as an indicative criterion of exposure to chemical stress (Wu and Lam, 1997 and Diamantino *et al.,* 2001). Similar results were also observed in different insects exposed to different insecticidal stress. In *culex sp*. after treatment with DDT, Malathion and cyfluthrin, LDH deacreased with highly dramatic ratios (Arshad *et al.,*2002). Nathan *et al*. (2005) showed that treatment of *S. littura* with azadirachtin highly decreased this enzyme in the mid gut. Similar results also were achieved in case of the rice striped stem borer treated with diazinon (Zibaee *et al.,* 2008).

Trehalase is the only enzyme capable of hydrolyzing trehalose to its glucose monomeric units (Temesvari and Cotter, 1997). Trehalase might be an interesting target in the development of new techniques controlling insects (Silva *et al.,* 2004). In many organisms, changes in trehalase activity are closely linked to alteration in physiological conditions or development, indicating that this enzyme plays an important role in such biological functions as homeostasis and developmental events (Temesvari and Cotter, 1997). Previous studies on carbohydrate metabolism during larval and pupal development of silkworms and blowfly have shown that glycogen which is stored in the fat body was released into the blood in the form of trehalose (Murphy & Wyatt,1965 and Clegg & Evans, 1961) and the trehalose to glucose by trehalase (Friedman, 1967). Since metabolic utilization of trehalose is dependent upon trehalase, the increase in the activity of trehalase may be due to higher metabolic utilization of trehalose reserves under induced insecticidal stress conditions (Friedman , 1978). Nath (2000) revealed a significant decrease in fat body glycogen on exposure to organophosphorus insecticides which supports our findings.

The overall conclusion indicated that Spinetoram effect upon the two tested field strains of *S. littoralis* larvae may offer some assumptions and hypothesis, (1) major detoxifying enzyme seem to be of no role in defensive mechanism against Spinetoram, (2) Spinetoram seems to work via mimicking ACh, thus it enhances the overproduction of AChE but it doesn't combat ACh responsible causing symptoms due to Spinetoram toxicity, (3) The attack of this bioinsecticide enhances the stored nutritional fuel to be released as if the insect suffers starvation.(4) In the most studied parameters, Behira Governorate was more sensitive and highly affected than Kalyobia one which again can be attributed to the specific characterization of each strain and prevalent environmental conditions.

We have to mention that the last decade has seen large advances in our understanding of the molecular basis of insecticide resistance. The structural genes coding for the enzymes, which are elevated in a number of insect species, have been cloned and characterized. Our understanding of how these genes are regulated will form another major advance in our understanding of such systems, moving us closer to the goal of manipulating pest insect species with the aim of restoring insecticide susceptibility.

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ARABIC SUMMARY

التغيرات فى الانزيمات المضادة للسمية وأيض الكربوهيدرات المصاحبة للسبينوترام في سلالتين حقليتين لدودة ورق القطن *سبودوبترا ليتولاريس*

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تمت هذة الدراسة بهدف التعرف على طرق الدفاع ضد المبيد الحيوى سبينوترام لدى سلالتين حقليتين لدودة ورق القطن *سبودوبترا ليتولاريس* تم جمعهم من محافظتين مصريتين هما القليوبية و البحيرة بالاضافة قياس تأثيرة على ايض الكربوهيدات.وقد ناقشت الدراسة الدور الذى تلعبة العديد من المعايير الكيموحيوية مثل قياس نشاط انزيمات الاستريزالعامة والانزيمات الناقلة للجلوتاثيون و قد أظهرت هذة الدراسة ان مثل هذة الانزيمات الدفاعية لا تلعب دورا فعالا ضد هذا المركب فيما عدا انزيم الاستيل كولين استريزالذى سجل ارتفاعا وصل الى 18.7% نتيجة الرش الحقلى لهذا المركب وقد سجلت الدراسة ايضا زيادة ملحوضة فى مادتة المتفاعلة وهى الاستيل كولين حيث زاد تركيزها الى 42.8% بينما الانزيمات والمركبات الخاصة بأيض الكربوهيدرات قد تأثرت بهذا المركب بشكل ملحوظ مثل الجليكوجين الذى هبط تركيزة الى 34.9% أما اللاكتات ديهيدروجينيزفقد نقص نشاطة حتى 55.7% بينما سجلت الدراسة نشاطا ملحوظا لانزيم التريهاليز وصل الى.22.8%. أظهرت النتائج أيضا محافظة البحيرة كانت أشد تأثرا من محافظة القليوبية.تعتبر هذة الدراسة تمهيدا فى فهم طريقة عمل هذا المركب و فهم ظاهرة المقاومة المتوقعه لهذا المبيد لدى السلالة الحقلية لهذة الحشرة .