

# Monitoring and biochemical impact of insecticides resistance on field populations of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) in Egypt

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**Abstract:** Cotton leafworm, *Spodoptera littoralis* (Boisd.) is a key pest affecting many field crops and vegetables in Egypt. Therefore, in the current study, the susceptibility of the 2<sup>nd</sup> instar larvae of *S. littoralis* laboratory strain to eight insecticides was investigated and the insecticide resistance levels of two field populations were monitored. The two populations were collected from two governorates in Egypt, namely El-Monufia (MS) and El-Fayoum (FS). Resistance monitoring showed that the field population from El-Fayoum was highly resistant to chlorantraniliprole, emamectin benzoate (EMB), spinotram, and spinosad. However, the El-Monufia field population only exhibited high resistance to chlorantraniliprole and spinosad. The relative toxicity showed that the laboratory strain is highly sensitive to EMB (LC<sub>50</sub>= 0.001 ppm) followed by spinotram (LC<sub>50</sub>= 0.006 ppm), chlorantraniliprole (LC<sub>50</sub>= 0.008 ppm), spinosad (LC<sub>50</sub>= 0.008 ppm), and indoxacarb (LC<sub>50</sub>= 0.021 ppm), while chlorfenapyr, fipronil, and alpha-cypermethrin exhibited low toxicity to the laboratory strain of *S. littoralis*. Moreover, the biochemical determinations of detoxification enzymes revealed that carboxylesterase ( $\alpha$ , and  $\beta$  -esterase), and AChE activity were significantly increased in the FS population. Thus, Glutathione S-Transferase (GST) showed significant increase in the two populations.

**Keywords:** monitoring, resistance, *Spodoptera littoralis*, insecticides, detoxification enzymes

## Introduction

Cotton leafworm, *Spodoptera littoralis* (Boisduval, 1833) is a widely spread pest in Egypt. It is the main cause of losses in more than 87 of many economically important crops such as cotton, corn, peanut and soybean (Pineda *et al.* 2007; El-Sheikh *et al.* 2018). As a result, different insecticide formulations from various classes of insecticides have been used in *S. littoralis* management. This intensive use of insecticides has caused the development of resistance to almost all the insecticide groups used (Abo *et al.* 2005). *S. littoralis* has developed resistance to 31 compounds (Arthropod Pesticide Resistance Database, 2021) including organophosphorus, pyrethroid and indoxacarb. As a result, insecticides with novel modes of action have

been introduced in Egypt over the last two decades to prevent or delay widespread resistance. However, worldwide insecticide resistance against one or more new chemistry insecticides, such as chlorantraniliprole, spinosad, emamectin benzoate and indoxacarb, have been expressed in different Noctuidae species, including *S. littoralis* (Rehan and Freed 2014; Ahmad *et al.* 2018), *Spodoptera litura* (Fabricius, 1775) , *Spodoptera exigua* (Hübner, 1808) (Huang *et al.* 2021) and *Plutella xylostella* (Linnaeus, 1758) (Tamilselvan *et al.* 2021).

Understanding the mechanisms of resistance, by measuring the activity of metabolic enzymes as a reliable biomarker, could be a useful tool in the management of insect resistance. The metabolic enzymes include mixed function oxidase (MFO), carboxylesterases (CarEs), and glutathione S-transferases (GSTs) (Mohan and Gujar

2003). The increased level of these detoxifying enzymes in insecticide-resistant populations is mostly responsible for global resistance (Gao & Shen 2011).

As a result of resistance evolution, the need for continuous monitoring of resistance frequencies in field strains of this pest has increased. Monitoring resistance allows decisions concerning the effective management strategies to be taken (Prabhaker *et al.* 1996). The pattern of resistance could be detected within the governorate or between governorates.

Consequently, this study aimed to assess resistance in two populations of cotton leafworm collected from two governorates, to conventional and new chemical insecticides. The activity of metabolic enzymes was also assessed to determine their possible role in resistance of the cotton leaf worm to insecticides.

## Materials and methods

### Insect Cultures

The laboratory strain (Ss) of *S. littoralis* was used as the reference population. The strain was reared in the laboratory according to El-Defrawi *et al.* (1964) with some modification (Moustafa *et al.* 2021). Two field populations

of *S. littoralis* were collected from El-Monufia (MS), and El-Fayoum (FS) governorates. The strains were collected and the 2<sup>nd</sup> instar larvae of the first generation were used for the bioassay and detoxification enzymes assay. The colonies were kept in a rearing room at 25±1°C, 75±5% relative humidity, 16L:8D (light: dark) photoperiod. Larvae were fed with fresh castor bean leaves, while moths were fed with a 10% sugar solution (Kandil *et al.* 2020).

### Insecticides and chemicals used

The insecticides used in the current study are spinosyns, emamectin benzoate, and chlorfenapyr as bio-insecticides. Chlorantraniliprole, indoxacarb, and fipronil were mentioned as newer insecticides and alpha-cypermethrin as traditional insecticides (Table 1). Acetylthiocholine iodide (ATChI), triton X-100, fast blue B, glutathione (GSH), p-nitroanisole (p-NA), 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma Aldrich. 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) was obtained from Roth, reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Sorachim, a-naphthyl acetate (a-NA) was obtained from Mpbio.

Table 1. Tested insecticides, and their mode of action.

Insecticides Common name		Trade names	Formulation a.i. %	The Manufacturer	Mode of Action*
Spinosyns	Spinosad	Tracer	24% SC	Dow Agro Sciences, UK	Nicotinic acetylcholine receptor (nAChR) allosteric modulators – Site I
	Spinetoram	Radiant	12% SC	Dow Agro Sciences, UK	
Emamectin benzoate		Proclaim	5% SG	Syngenta Agro. Switzerland	Glutamate-gated chloride channel (GluCl) allosteric modulators
Chlorfenapyr		Challenger	24% SC	BASF, USA	Uncouplers of oxidative phosphorylation via disruption of the proton gradient
Chlorantraniliprole		Coragen	20% SC	DuPont, France	Ryanodine receptor modulators
Indoxacarb		Avaunt	15% EC	DuPont, France	Voltage-dependent sodium channel blockers
Fipronil		Coach	20% SC	Star chem. Egypt	GABA-gated chloride channel blockers
Alpha-cypermethrin		Acta	10% EC	BASF, USA	Sodium channel modulators

\* Insecticide Resistance Action Committee (IRAC, 2020)

### Bioassays

Bioassays were performed on 2<sup>nd</sup> instar larvae of *S. littoralis* for laboratory (Ss) and field (MS, and FS) strains using the leaf dipping technique according to Moustafa *et al.* (2021) with some modification. For each insecticide evaluated, castor bean leaves were dipped in six different concentrations for 20 s. The treated leaves were allowed to dry, then placed into a glass jar (0.25 L) with 10 larvae. There were five jars per concentration. Untreated leaves served as the control treatment. The larvae were allowed to feed on the treated leaves before being transferred onto untreated leaves. LC<sub>50</sub> and LC<sub>90</sub> values of each insecticide were calculated after 96 hours post-treatment. The bioassay was repeated twice.

### Detoxification enzymes

#### Sample preparations

Strains of Ss, MS and FS of the 2<sup>nd</sup> instar larvae of *S. littoralis* were kept at -20 °C for the biochemical assays. 0.1g of larvae were homogenized in 0.1 M phosphate buffer with different pH values according to the enzyme in ratio 1:10 (W: V). For each enzyme assay, five replicates per strain were used to record the mean enzyme activities in each strain.

#### Mixed Function Oxidases (MFO)

MFO activity was tested according to Hansen and Hodgson (1971), where larvae were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.8) and centrifuged at 15,000g for 15 min at 4 °C. 100 µl of 2 mM p-nitroanisole with 90 µL enzyme stock solutions were added to each well of a microplate and mixed. After incubation for 2 min at 27 °C, the 10 µL of 9.6 mM NADPH was added to initiate the reaction. The activity of MFO was measured immediately at 405 nm for 15 min using molecular devices of Vmax kinetic microplate reader. A standard curve of p-nitrophenol was used to calculate the MFO activity.

### $\alpha$ - and $\beta$ - esterase assay

Esterase activity was examined according to Van Asperen, (1962) with some modifications (Moustafa *et al.*, 2021). Larvae were homogenized in phosphate buffer (0.1 M, pH 7.0) and centrifuged at 12,000g for 15 min at 4 °C. 50 µl enzyme solution was incubated with 50 µl of  $\alpha$  or  $\beta$ - NA (30 mM) for 15 min at 30 °C. To stop the reaction, two parts of 1% Fast Blue B and five parts of 5% sodium dodecyl sulfate were added. The absorbance was measured at 600 nm for the hydrolysis of  $\alpha$ -NA and 550 nm for  $\beta$ - NA at UV/Vis Spectrophotometer (Jenway 7205UV/Vis). Mean levels of total esterase activity were based on protein content and  $\alpha$  and  $\beta$ - naphthol standard curves.

### Glutathione S- transferase assay

The glutathione-S-transferase activity was measured as indicated by Habig *et al.* (1974). Larvae were homogenized in ice-cold phosphate buffer (0.1 M, pH 6.5) and centrifuged at 12,000g for 15 min at 4 °C. The reaction solution contained 10 µl supernatant, 25 µl of 30 mM CDNB, and 25 µl of 50 mM GSH. The activity was determined by continuous monitoring of the change in absorbance at 340 nm for 5 min using a UV/Vis Spectrophotometer (Jenway 7205UV/Vis).

### AChE assay

Acetylcholine esterase (AChE) activity was measured according to Ellman *et al.* (1961) with modifications (Fouad *et al.* 2016). Larvae were homogenized in an ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.1% (V/V) Triton X-100 then centrifuged at 13,000g at 4 °C for 15 min. 800 µl potassium phosphate buffer (0.1 M, pH 7.5) was added to 100 µl enzyme solution, 50 µl of 0.075 M ATChI and 50 µl of 0.01 M DTNB. The change in absorbance at 412 nm was recorded for 5 min using a UV/Vis spectrophotometer (Jenway 7205UV/Vis).

### Statistical analysis

The corrected mortality percentages were statistically analyzed according to the method of Finney (1971) by using Log Dose Probit (LDP) Line software to estimate the values of  $LC_{50}$  and  $LC_{90}$  of tested insecticides on 2<sup>nd</sup> instar larvae of different strains of *S. littoralis* after four days post-treatment. The resistance ratio (RR) was calculated by dividing the  $LC_{50}$  value of a field strain by the  $LC_{50}$  value of the Laboratory strain. Levels of resistance were classified according to Ahmad & Iqbal Arif (2009): susceptible (RR  $\leq$  1-fold), very low resistance (RR= 2-10 fold), low (RR = 11-20) moderate resistance (RR= 21-50), high resistance (RR= 51-100) and very high (RR > 100). Detoxification enzyme activity was statistically performed using one-way ANOVA (the df., F and P-values were established) included SAS software (SAS, 2001). The values were analyzed using Duncan's multiple range test.

## Results

### Toxicity of tested insecticides to the susceptible strain

Based on the presence of overlap in the 95 % FLs, the results of bioassays for bioinsecticides against the susceptible strain revealed that the toxicity of spinosad ( $LC_{50}$ = 0.008 mg/L) was similar to that of spinotram ( $LC_{50}$ = 0.006 mg/L). Emamectin benzoate was the most toxic of the bioinsecticides tested, with an  $LC_{50}$  of 0.001 mg/L, while chlorfenapyr was the least toxic, with an  $LC_{50}$  of 20.14 mg/L (Table 2).

Chlorantraniliprole was the most toxic of the chemistry insecticides tested, followed by indoxacarb with  $LC_{50}$  values of 0.008 and 0.021 mg/L, respectively. Fipronil was the least toxic of the insecticides tested, with an  $LC_{50}$  of 19.09 mg/L. The laboratory strain of

*S. littoralis* was moderately susceptible to alpha-cypermethrin, a conventionally tested insecticide, with an  $LC_{50}$  of 13.95 mg/L. (Table 2).

### Toxicity of bio-insecticides to field populations

The resistance ratios (RR) for spinosad in MS and FS populations were 108.75- and 316.25-fold compared to the susceptible strain. The resistance to spinotram in *S. littoralis* was low in the MS population (0.44-fold), but it was 106.7-fold in the FS population (Table 2).

The MS population had a 3-fold resistance to emamectin benzoate, while the FS population had a 90-fold resistance. In the case of chlorfenapyr, the resistance of *S. littoralis* was the lowest, with 0.25- and 1.04-fold in MS and FS, respectively. (Table 2).

### Toxicity of new chemistry insecticides and conventional insecticide to field populations

Results of the toxicity of newer chemistry insecticides *i.e.*, Chlorantraniliprole, indoxacarb, and fipronil against MS and FS populations are shown in Table 2. When compared with the susceptible strain, the resistance ratios of Chlorantraniliprole were 86.25- and 55-fold in MS and FS populations, respectively. *S. littoralis*, on the other hand, demonstrated low levels of resistance to indoxacarb and fipronil, with RRs of 10.48- and 3.67-fold for indoxacarb, and 0.5- and 0.39-fold for fipronil in the MS and FS populations, respectively. Although Alpha-cypermethrin is a conventional insecticide, *S. littoralis* demonstrated a low level of resistance, with RRs of 2.55- and 0.99-fold in the MS and FS populations, respectively. (Table 2).

Table 2. Toxicity of bioinsecticides, new chemistry, and traditional insecticides against two field populations of *Spodoptera littoralis* from Egypt.

Insecticides	Strains	No.	LC <sub>50</sub> (µg ml <sup>-1</sup> ) (95% CL)	Slope (SE)	χ <sup>2</sup>	g value	RR
Spinosad	S	80	0.008 (0.002-0.021)	0.74 (0.18)	0.58	0.24	
	M	90	0.87 (0.68-1.03)	2.00 (0.32)	0.41	0.19	108.75
	F	100	2.53 (1.72-5.06)	1.57 (0.41)	0.26	0.27	316.25
Spinotram	S	90	0.006 (0.001-0.014)	0.73 (0.19)	1.39	0.25	
	M	80	0.28 (0.14-0.52)	1.19 (0.27)	1.62	0.19	0.44
	F	150	0.64 (0.42-1.32)	1.17 (0.27)	0.98	0.20	106.7
Emamectin benzoate	S	70	0.001 (0.0004-0.003)	0.98 (0.22)	0.97	0.19	
	M	110	0.003 (0.002-0.007)	1.02 (0.22)	0.04	0.18	3
	F	80	0.09 (0.04-0.56)	0.93 (0.23)	0.12	0.24	90
Chlorfenapyr	S	70	20.14 (12.31-27.17)	2.47 (0.66)	0.01	0.28	
	M	90	5.04 (4.25-5.88)	2.72 (0.25)	0.11	0.03	0.25
	F	110	20.90 (13.96-34.94)	1.57 (0.37)	0.47	0.24	1.04
Chlorantraniliprole	S	118	0.008 (0.003-0.017)	0.81 (0.13)	1.67	0.10	
	M	120	0.69 (0.33-1.04)	1.53 (0.34)	0.65	0.19	86.25
	F	80	0.44 (0.13-0.99)	0.85 (0.21)	0.31	0.26	55
Indoxacarb	S	110	0.021 (0.012-0.035)	1.24 (0.23)	0.93	0.13	
	M	80	0.22 (0.13-0.34)	1.76 (0.31)	0.04	0.12	10.48
	F	80	0.077 (0.036-0.178)	1.10 (0.19)	0.65	0.15	3.67
Fipronil	S	70	19.09 (14.59-23.94)	3.54 (0.73)	0.06	0.16	
	M	50	9.52 (3.32-17.48)	1.53 (0.41)	0.11	0.28	0.5
	F	70	7.47 (5.77-11.57)	3.12 (0.74)	0.01	0.22	0.39
Alpha-cypermethrin	S	150	13.95 (11.17-17.53)	2.6 (0.36)	0.31	0.07	
	M	90	35.53 (25.68-50.22)	2.25 (0.43)	1.24	0.14	2.55
	F	120	13.83 (10.40-17.69)	2.38 (0.42)	1.05	0.12	0.99

### Detoxification enzyme activities

Enzyme assays were carried out to measure the levels of mixed function oxidase (MFO), carboxylesterases ( $\alpha$  and  $\beta$ - esterases), glutathione-S-transferase (GST), and acetylcholine esterase (AChE) in the laboratory susceptible strain (Lab- S) and the

two field-collected populations (MS and FS) in order to determine the probable role of detoxification enzymes in causing the variable susceptibility to the tested bioinsecticides, newer chemistry, and conventional insecticides (Table 3).

Table 3. Mean ( $\pm$ SE) of detoxification enzymes activity in *Spodoptera littoralis* strains.

Strains	Mean $\pm$ SE				
	MFO ( $\mu$ mole/min/mg of protein)	Carboxylesterases (mmole/min/mg of protein)		GST (mmole/min/mg of protein)	AChE (mmole/min/mg protein)
		$\alpha$ -esterase	$\beta$ -esterase		
Susceptible	19.55 <sup>a</sup> $\pm$ 0.19	7.72 <sup>b</sup> $\pm$ 0.12	5.62 <sup>b</sup> $\pm$ 0.31	0.049 <sup>c</sup> $\pm$ 0.0007	0.013 <sup>b</sup> $\pm$ 0.0015
El-Monufia	19.36 <sup>a</sup> $\pm$ 0.20	10.1 <sup>a</sup> $\pm$ 0.31	7.58 <sup>a</sup> $\pm$ 0.29	0.064 <sup>b</sup> $\pm$ 0.0005	0.021 <sup>a</sup> $\pm$ 0.0015
El-Fayoum	16.66 <sup>b</sup> $\pm$ 0.08	6.43 <sup>c</sup> $\pm$ 0.30	5.13 <sup>b</sup> $\pm$ 0.09	0.091 <sup>a</sup> $\pm$ 0.0039	0.009 <sup>c</sup> $\pm$ 0.0009
F	90.55	51.47	26.75	79.16	51.75
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Data are shown as the mean  $\pm$  SE followed by the same letter in a column are not significantly different at  $P < 0.05$  by Duncan's multiple range test.

Mixed function oxidase activities varied little across lab-S and MS populations, with 19.55 and 19.36  $\mu$ mol/min/mg protein, respectively. The FS population had the lowest MFO activity (16.66  $\mu$ mol/min/mg protein).

The  $\alpha$ - esterase activity of the MS population (10.1 mmol/ min/ mg protein) was significantly higher than that of the lab-S population (7.72 mmol/ min/ mg protein). The FS population had the lowest  $\alpha$ - Esterase activity, which was 6.43 mmol/ min/ mg protein.

The  $\beta$ -esterase activity of the Lab-S and FS populations were not significantly different, with 5.62 and 5.13 mmol/min/mg protein, respectively. The MS strain had the highest significant increase in  $\beta$ - esterase activity, which was 7.58 mmol/ min/ mg protein.

The GST activities of the two field populations (MS and FS) were higher than those of the lab susceptible strain, but the increase was significantly greater in the FS population than in the MS population. In MS, FS, and Lab-S, the GST activities were 0.064,

0.091, and 0.049 mmole/min/mg of protein, respectively.

The field collected population, MS, expressed higher significant level of acetylcholine esterase activity than the other field collected population, FS, as they were 0.021 and 0.0009 mmole/min/mg protein, respectively. Apart from that, the MS population showed a less significant rise in AChE activity than the reference susceptible strain, which had 0.013 mmole/min/mg protein.

### Discussion

To date, chemical insecticides remain the primary approach for *S. littoralis* management, and the important groups of insecticides used for *S. littoralis* control in crops in Egypt are emamectin benzoate, diamides, indoxacarb, Spinetoram, organophosphorus, pyrethroids, IGRs, and Bt (Egyptian Agricultural Pesticides Committee 2020). In contrast, the development and spread of insecticide resistance could reduce their efficiency, so regular monitoring of

insecticide resistance is essential to provide information on the status of insect field populations' resistance and ensure effective management.

The current study investigated the susceptibility of 2<sup>nd</sup> instar of *S. littoralis* laboratory strain to eight insecticides with a different mode of action, and the insecticides resistance was also evaluated for two field populations of *S. littoralis*. Results showed that emamectin benzoate was highly efficient against the 2<sup>nd</sup> instar larvae followed by Spinosyn (spinosad and spinetoram) as bioinsecticides, chlorantraniliprole observed the same LC<sub>50</sub> value of spinosad. On the other hand, Fipronil and alpha-cypermethrin exhibited lower toxicity against the laboratory strain of *S. littoralis*. These results are congruent with that of Tamilselvan *et al.* (2021) who found that spinetoram, spinosad, emamectin benzoate, and chlorantraniliprole were more toxic to a susceptible population of *P. xylostella*, while indoxacarb and cypermethrin were the least toxic.

FS exhibited a very high resistance to spinosad followed by spinotram and high resistance to emamectin benzoate and chlorantraniliprole. A high level of resistance to spinosad and chlorantraniliprole against *S. littoralis* was detected in MS, while indoxacarb demonstrated a low level of resistance in MS.

Resistance to spinosad, emamectin benzoate, Chlorantraniliprole and indoxacarb has been reported in *P. xylostella* (Zhang *et al.* 2016; Tamilselvan *et al.* 2021). *S. exigua* exhibited resistance to spinosad (Ishtiaq *et al.* 2014), indoxacarb, emamectin benzoate, and chlorantraniliprole (Huang *et al.* 2021). Additionally, resistance to diamide insecticides has already been reported in lepidopteran species (Pereira *et al.* 2020). Thus, resistance to emamectin benzoate and spinosad had been reported in *S. litura* (Rehan & Freed 2014; Zaka *et al.* 2014; Wang *et al.* 2019).

Metabolic enzymes such as mixed-function oxidase, carboxylesterases, and glutathione S-transferase are essential elements in the

development of insecticide resistance. In the current study, the resistance to chlorantraniliprole, spinosad, and indoxacarb are associated with an increase in the activity of glutathione S-transferase (GST), carboxylesterases, and acetylcholine esterase in MS populations; glutathione S-transferase (GST) is the only enzyme which exhibited higher activity in FS associated with resistance to Chlorantraniliprole, spinosad, spinotram, and emamectin benzoate. In addition, the activity of mixed-function oxidase did not significantly increase in the two populations. These results are in agreement with Hu *et al.* (2014) and Zhang *et al.* (2016), who found that the overexpression of glutathione S-transferase and carboxylesterase (CarE) is related to resistance to diamide insecticides. Additionally, there is also positive correlation between resistance to spinosad and increasing the activity of glutathione S-transferase and carboxylesterases enzyme (Gong *et al.* 2013). The carboxylesterase and GST were major factors leading to indoxacarb resistance in *S. exigua* and *P. xylostella* (Sayyed & Wright 2006; Nehare *et al.* 2010; Gao *et al.* 2014). Thus, the increased activities of carboxylesterase and MFO are conferred indoxacarb resistance in *S. litura* (Wang *et al.* 2019). In contrast, Gong *et al.* (2013) and Khan *et al.* (2016) found no correlation between enzyme activities and emamectin resistance in *P. xylostella*.

Further studies have confirmed cross-resistance between the spinosad and indoxacarb with chlorantraniliprole (Zhang *et al.* 2016; Fu *et al.* 2018; Tamilselvan *et al.* 2021). In addition, a lack of cross-resistance between indoxacarb and emamectin benzoate has been reported in resistant strain of *S. litura* (Shad *et al.* 2010; Ishtiaq *et al.* 2014). The cross-resistance between different groups of insecticides might be due to the metabolic detoxification mechanisms (von Stein *et al.* 2013). Therefore, it is necessary to develop effective management plans to delay further resistance development and product failure. Thus, to identify the

effectiveness of insecticides for *S.* management, a standard resistance monitoring is needed.

## Conclusion

According to this study, *S. littoralis* can develop resistance to bioinsecticides and new chemical insecticides, owing to increased GST and carboxylesterase detoxification. The findings are a first step toward a better understanding of the biochemical mechanisms of the tested insecticide resistance in lab and field strains. More research is needed to confirm the resistance mechanism and to develop more effective management strategies for *S. littoralis* based on the inheritance patterns of tested insecticide resistance.

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