Original Article

Cholinesterase and Aliesterase as a Natural Enzymatic Defense against Chlorpyrifos in Field Populations of *Spodoptera Littoralis* (Boisdüval, 1833)(Lepidoptera, Noctüidae)

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ABSTRACT

In insecticide resistance research on cotton leafworm multiple resistance mechanisms, including behavioral resistance, some types of physiological resistance (e.g. increasing activities of esterases), were identified as conferring organophosphate insecticide resistance in *Spodoptera littoralis* (Boisdüval, 1833) after decades used in Egypt. Enzyme kinetic parameters (K_m and V_{max}) and *in vitro* inhibition were used to detect variations in Cholinesterase (ChE) and Aliesterase (Ali-E) activities among different field populations and determine tolerance levels of field populations to chlorpyrifos using the dipping technique. Results revealed that, *S. littoralis* resistance levels in fourth-instar larvae exposed to chlorpyrifos were 9- to 120-fold in field populations compared with an insecticide-susceptible population. Activity of esterase preparations of larval head homogenates varied, that is the relative activity of ChE and Ali-E were 1.71 and 4.23-fold respectively in heavily-sprayed field populations while recorded 1.27 and 2.53-fold in desert field populations. The kinetic studies revealed that the Michaelis Constant (K_m) and Maximal velocity (V_{max}) values of ChE and Ali-E were higher in all field populations than susceptible population. The affinity of ChE, toward alpha-naphthyl acetate (α-NA) as a substrate, in *S. littoralis* was higher than that of Ali-E in all field populations. The ChE and Ali-E level was higher in the field-populations than its level in susceptible-population. Results revealed that the Ali-E was much more sensitive to *in vitro* inhibition by chlorpyrifos than ChE in field populations, meaning that ChE had an antagonistic effect on chlorpyrifos.

1. Introduction

Since synthetic insecticides were introduced to control pests, increased resistance to widespread insecticides has been shown in more than 540 species of insects, as a result of selective pressure by insects, and the excessive use in the fields [1]. According to the United States Environmental Protection Agency (USEPA), over the past three decades, more than 2.5 million tons of pesticides have been applied to protect agricultural crops every year, and overcome the damage caused by insect resistance to pesticides, exceeding $ 100 billion annually [2].

Several studies have found that developing insecticide resistance mechanisms in most insect species is a major problem in pest control. Insects can resist insecticides through many physiological mechanisms, including interfering with enzyme systems by producing

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detoxifying enzymes and/or effects on the target sites of the pesticide, rendering them insensitive. Besides, the participation of insect esterases in their tolerance to insecticides with variation between a member of the esterase such as aliphatic esterases, phosphatases, non-specific esterases is likely to play a pivotal role in the detoxification mechanisms of insecticides and increase resistance in insect [3-5].

Increased detoxification by esterases is a common mechanism of Organophosphorus (OPs)–insecticide resistance in insects. This mechanism has stimulated research on the role of esterases in different directions, particularly in OPs–insecticides, a group of insect cholinesterase inhibitors, which are currently widely used to protect various crops from insect damage [6-9].

The Egyptian cotton leafworm *Spodoptera littoralis* (Lepidoptera, Noctüidae) is an economically important and polyphagous pest, which seriously damages more than 60 different field crops and consequently reducing quantity and quality of the yield [1]. Cotton, the most important economic crop in Egypt, is liable to be attacked by different pests; one of the most injurious is the cotton leafworm [10]. Because of the wide application of synthetic insecticides in the field several times a year, resistant to this pest have been recorded from time to time to different groups of insecticides in different regions. Increasing insect resistance to various insecticides leads to difficult pest management tasks in the agriculture field [11]. Therefore, the knowledge of the type of resistance specters of the resistant populations may offer valuable information in the search for new compounds to be used instead of those which have lost their toxic effect on resistant populations. Also, this may contribute to improving insecticide recommendations for controlling resistant insects of medical or agricultural importance. Hence, the information associated with susceptibility in cotton leafworm is essential for effective pest management programs. Correlation between high esterase activity and insecticide resistance was reported in *S. littoralis* [12, 13].

There is a relationship between Aliesterase (Ali-E), Cholinesterase (ChE) levels and resistance of *S. littoralis* to insecticides [14]. Hence, the present study was conducted in order to investigate variations of some esterases (ChE and Ali-E) activities among different field populations (from heavily-sprayed fields and desert fields) of *S. littoralis* larvae. In addition, tolerance levels of *S. littoralis* populations were determined to chlorpyrifos, one of the OPs insecticides in current use in Egyptian fields to control this pest.

2. Materials and Methods

2.1. Insect source

A susceptible strain of the cotton leafworm is *S. littoralis* Boisdüval (Boisdüval, 1833) (Lepidoptera: Noctüidae). This strain was continuously reared on castor bean leaves of *Ricinus communis* Linnaeus, 1753 for several years without exposure to any pesticides at the Department of Insect Population Toxicology, Central Agricultural Pesticides Laboratory, Agriculture Research Center, Dokki, Giza, Egypt, under controlled laboratory conditions at a temperature of 25 ± 1 °C with 65 ± 5% relative humidity with a 16:8-h light: dark photoperiod "laboratory susceptible population" (Figure 1).

The *S. littoralis* samples respective regions during the period from to 4th April, and 26th of Jun, 2020 were collected randomly as heavily infested cotton leaves with egg masses from fields heavily-sprayed districts Biala, Abou El-Matamir and recently cultivated desert fields, El-Salhia district of Kafr El-Sheikh, El-Beheira and Alexandria Governorates in Egypt. All field populations were exposed to various insecticides in the field. The Biala and Abou El-Matamir fields were sprayed several times with OPs–insecticides. The El-Salhia were desert fields, so we could not ascertain the insecticide exposure history of these populations accurately. The *S. littoralis* egg masses were collected and carefully transferred to the laboratory under the same previous conditions until the hatched larvae and then were reared on castor bean leaves. The fourth instar larval

was used in all experiments in the present investigation[15].

Figure 1. Egyptian cotton leafworm *Spodoptera littoralis* and castor bean leaves (*Ricinus communis* L.).

2.2. Bioassays

The leaf-dip method was used to assess the toxicity of commercial–grade chlorpyrifos 48% EC. Homogenous pieces of the castor beans leaves were dipped in six concentrations of chlorpyrifos for 10 sec., held vertically to allow the excess solution to drip off and dried at room temperatures. Treated castor bean leaf pieces were transferred to plastic cup, and thirty starved larvae (4th instar) were added to each concentration in triplicate. Treated as well as control larvae were maintained in the laboratory at a temperature of 25-±-1-°C with 65-±-5% relative humidity with 16:8-h light: dark photoperiod. The mortality was recorded 24 h after treatment.

2.3. Biochemical analysis of enzyme assays

2.3.1. Preparation of head homogenate

Twenty early fourth instar larvae from different field populations including laboratory susceptible population larvae were separated, starved for 24 hr and then cut to heads. The insect head was homogenized in 2.5 mL phosphate-buffered saline (0.1 M phosphate-buffered saline, pH 7.6) on ice-cold condition. The homogenate was centrifuged at 4 ° C, 5,000 rpm for 30 min; solid debris and cellular material were removed. The supernatant was collected in a sterile, refrigerated container, placed on ice, and used immediately for assaying Cholinesterase (ChE) and Aliesterase (Ali-E).

2.3.2. Cholinesterase and Aliesterase assay

The Cholinesterase and Aliesterase activities were determined to homogenize the head using alpha-naphthyl acetate (α-NA) as substrate. The insect head homogenate was prepared in triplicate. Ali-E activity was estimated in the presence of 10^{-6} M eserine salicylate as a specific inhibitor of ChE, according to the method [16]. The specific activity of ChE and Ali-E was calculated as μ mole α-NA hydrolyzed per mg protein per min at 30 °C. The rate of color production was measured as a function of enzyme activity at λ_{412nm}. The protein content of the head homogenates was determined by the method [17], and BSA was used as a standard protein.

2.3.3. *In vitro* enzyme inhibition

In vitro enzyme inhibition experiments of ChE and Ali-E were determined in head homogenates taken from chlorpyrifos treatment with a value of LC_{50} for each population. Ten microliters of head homogenate supernatant were incubated with 5mM inhibitor at 27 °C for 30 min. According to the method [16], 10^{-6} M eserine salicylate was used to inhibit ChE activity, thus allowing determination of Ali-E activity alone. After incubation, the absorbance value was recorded at λ_{412nm} then percentage of inhibition was estimated. Enzyme kinetics parameter, Michaelis constant (K_m) and maximal velocity (V_{max}) were calculated from linear regression of

6 points on each [18]. Experiments were performed in triplicates to minimize the error.

2.4. Statistical analysis

The data were analyzed using SPSS software (Version 10.0 for windows, SPSS Inc., Chicago, USA) to determine median lethal concentrations (LC_{50}). The data was expressed as a mean of replicates ± SE. Significant differences between enzyme activities were analyzed by using Tukey's multiple comparison test at $P< 0.05$.

3. Results

3.1. Bioassay

The bioassay tests of this study showed that chlorpyrifos in the laboratory susceptible population was more toxic than all field populations. The detailed toxicity effect of chlorpyrifos is tabulated in Table 1. Compared with the laboratory susceptible population, the highest tolerance level was found in the Kafr El-Sheikh Population 120-fold, followed by the El-Beheira population 70-fold, while the lowest was found in Alexandria's population at 8.96-fold. The slope values ranged from 0.57 to 2.88 in the field populations, indicating considerable heterogeneity in their responses to organophosphates. Slope values suggested a greater potential for developing high levels of resistance.

Table 1. LC_{50} value of *Spodoptera littoralis* larvae from the field- and susceptible-populations.

Spodoptera-Populations	LC_{50} (ppm) (95% Confidence limits)	Slope
Laboratory susceptible strain	10.03 (6.57-12.44)	1.97
Alexandria Governorate	89.87 (76.56-104.76)	2.88
El-Beheira Governorate	699.81 (518.45-959.44)	0.57
Kafr El-Sheikh Governorate	1205.67 (850.27-1917.95)	1.37

3.2. Cholinesterase and Aliesterase activities

The results in Table 2 show the activity averages of Total-E, ChE and Ali-E of fourth instar larvae of different field populations and laboratory susceptible populations. The results show significant differences in the specific activities of each group of Total-E, ChE and Ali-E among all the *Spodoptera* populations. In general, *S. littoralis* field populations displayed significantly higher levels of Total-E, ChE and Ali-E activity than laboratory susceptible strain Figure 2. This observation was relatively higher in the case of Kafr El-Sheikh population and followed in a descending order by the El-Beheira population. These two populations were from heavily-sprayed agricultural areas in Egypt. The recorded rates increase in the specific activity of Total-E, ChE and Ali-E in Kafr El-Sheikh population were almostly 2.27, 1.71 and 4.23 times of corresponding values of laboratory susceptible population, and in case of El-Beheira population, these rates were almost 1.86, 1.37 and 3.59 times of corresponding values of laboratory susceptible population. The Alexandria population had lower Total-E, ChE and Ali-E activities than those in the Kafr El-Sheikh and El-Beheira populations but higher these esterases activities than those in the laboratory susceptible population (Figure 3).

Table 2. Esterase activity levels in the field- and susceptible-populations of the *Spodoptera littoralis* as determined in the larvae head.

Spodoptera-Populations	Specific activity (µ mole α-NA hydrolyzed per mg protein per min at 30 °C)		
	Total-E	ChE	Ali-E
Laboratory susceptible strain	$2.98^{a} \pm 0.11$	$2.32^{a} \pm 0.10$	$0.66^{a} \pm 0.11$
Alexandria Governorate	$4.61^{b} \pm 0.13$	$2.94^{b} \pm 0.12$	$1.67^{b} \pm 0.14$
El-Beheira Governorate	$5.55^{c} \pm 0.17$	$3.18^{b} \pm 0.29$	$2.37^{b} \pm 0.13$
Kafr El-Sheikh Governorate	$6.76^{d} \pm 0.19$	$3.97^{c} \pm 0.27$	$2.79^{c} \pm 0.11$

Means ± SE followed in the same column by the same letter are not significantly different ($P< 0.05$; Tukey's HSD test).

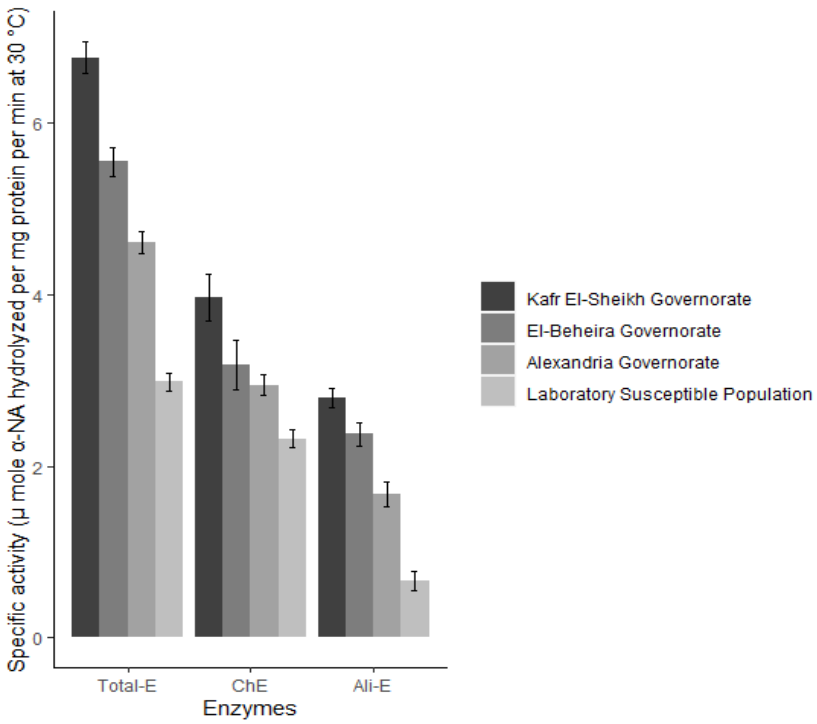

Figure 2. Levels of Esterase (± SE) in the field- and susceptible-populations of the *Spodoptera littoralis* as determined in the larvae head.

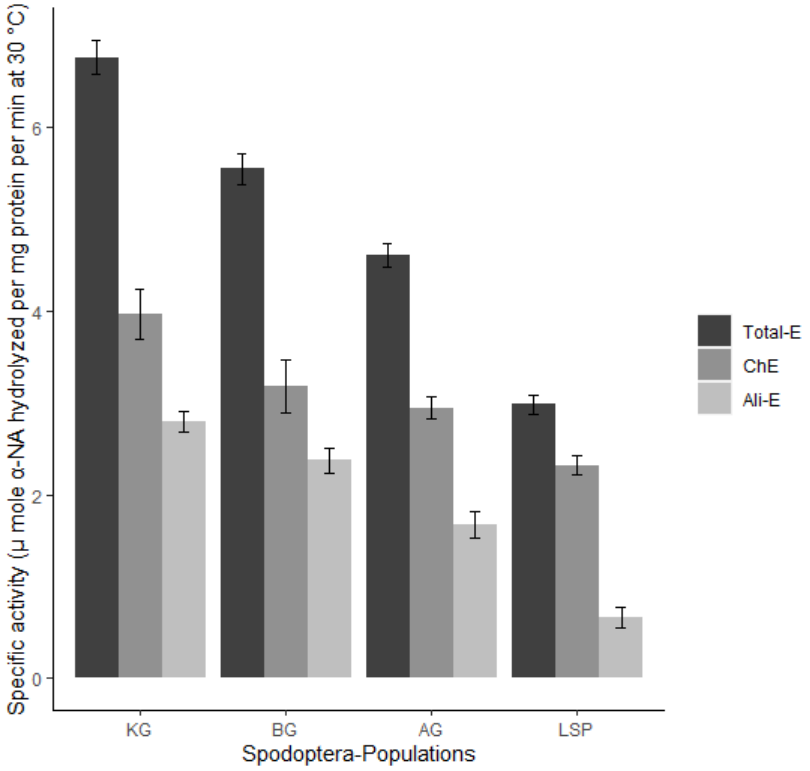

Figure 3. Esterase activity levels (± SE) in the field- and susceptible-populations of the *Spodoptera littoralis* as determined in the larvae head.

*KG= Kafr El-Sheikh Governorate, *BG= El-Beheira Governorate, *AG= Alexandria Governorate and *LSP= Laboratory Susceptible Population.

3.3. Kinetic constants

Table 3 showed the K_m and V_{max} of the Total-E, ChE and Ali-E of *S. littoralis* 4th instar larval head homogenates of the three tested field-populations as well as the laboratory susceptible-population. The K_m and V_{max} values for ChE and Ali-E were generally higher in all field-populations than in laboratory susceptible- population. The Km values both of ChE and Ali-E for Kafr El Sheikh Population about 4.20 and 3.52-folds the laboratory susceptible population, respectively, followed by El-Beheira population about 3.35 and 2.33-folds. The lowest values for both enzymes were recorded by the Alexandria population, which were 2.40 and 2.02-folds, respectively. The maximum velocity of the enzyme reaction was higher in all field populations than in the laboratory susceptible population.

Table 3. K_m and V_{max} values for Esterase from the field- and susceptible-populations of the *Spodoptera littoralis* larvae heads

Spodoptera-Populations	K_m (µM)			V_{max} (µM)		
	Total-E	ChE	Ali-E	Total-E	ChE	Ali-E
Laboratory susceptible strain	0.247^{a}±0.03	0.169^{a}±0.48	0.275^{a}±0.48	0.439^{a}±0.07	0.238^{a}±0.54	0.196^{a}±0.42
Alexandria Governorate	0.535^{b}±0.57	0.406^{b}±0.33	0.555^{b}±0.33	1.025^{b}±0.91	0.537^{b}±0.99	0.371^{b}±0.38
El-Beheira Governorate	0.721^{c}±0.19	0.566^{c}±0.27	0.640^{c}±0.27	1.380^{c}±0.92	0.737^{c}±0.63	0.500^{c}±0.47
Kafr El-Sheikh Governorate	0.988^{d}±0.13	0.709^{d}±0.10	0.967^{d}±0.10	1.444^{d}±0.12	0.767^{d}±0.77	0.594^{d}±0.98

Means ± SE followed in the same column by the same letter are not significantly different (P< 0.05; Tukey's HSD test).

3.4. *In vitro* inhibition enzyme

The figures in Table 4 indicated that there were differences between the laboratory susceptible population and the three field populations as regards Total-E, ChE and Ali-E sensitivity to inhibition by chlorpyrifos treatment. Ali-E was more sensitive to inhibition by chlorpyrifos than Total-E and ChE in the laboratory susceptible population and the three–field populations. The Ali-E activity of the Kafr El-Sheikh population was the least sensitive to inhibition by chlorpyrifos (I_{50} = 5 x 10^{-4} M) while this enzyme was most sensitive to inhibition by chlorpyrifos in the laboratory susceptible population followed by Alexandria population (I_{50} = 8 x 10^{-7} and 2 x 10^{-6} M, respectively). Regarding ChE inhibition, Table 4 shows that I_{50} of the enzyme in the Kafr El-Sheikh population (I_{50} = 9 x 10^{-8} M), represents the lowest sensitivity level for inhibition of ChE. On the other hand, the smallest I_{50} value of Total-E, ChE, and Ali-E was obtained with the laboratory susceptible population followed by the Alexandria population.

Table 4. I_{50} values of Esterase from the field- and susceptible- populations of the *Spodoptera littoralis* larvae heads.

Spodoptera-Populations	I_{50} (M)		
	Total-E	ChE	Ali-E
Laboratory susceptible strain	3x10^{-8a} ±0.15	8x10^{-7a} ±0.24	1x10^{-9a} ±0.24
Alexandria Governorate	7x10^{-8b} ±0.14	2x10^{-6b} ±0.11	5x10^{-9b} ±0.12
El-Beheira Governorate	4x10^{-6c} ±0.28	9x10^{-5c} ±0.17	3x10^{-8c} ±0.32
Kafr El-Sheikh Governorate	9x10^{-6d} ±0.23	5x10^{-4d} ±0.33	9x10^{-8d} ±0.16

Means ± SE followed in the same column by the same letter are not significantly different (P< 0.05; Tukey's HSD test).

4. Discussion

Esterases are involved in resistance to organophosphate compounds (OPs) widely used to control cotton leafworm populations. The ChE and Ali-E enzymes proved to be the most sensitive esterases to the action of OPs [19, 20] demonstrated the difference between susceptible and field strains in esterases activity with low sensitivity to *in vitro* inhibition by OPs in field strains. Therefore, the present study was conducted in order to investigate variations of some esterases activities (ChE and Ali-E) among different field populations of *Spodoptera littoralis* larvae. Also, the tolerance levels of the *S. littoralis* populations were determined against chlorpyrifos, one of the OP insecticides currently used in Egyptian fields to control this pest. Information on the variation in these esterase activities will be required to understand the mechanisms of resistance to this species and overcome insecticide resistance in the field.

S. littoralis is a phytophagous pest that causes critical economic losses in crops, and insecticides have been applied heavily to control this pest; however, the development of insecticide resistance, especially conventional insecticides (organophosphates, carbamates, and pyrethroids), has led to a failure to control this pest in the field [5]. Resistance to OP-insecticide was examined in field populations of *S. littoralis* collected from different field populations. *S. littoralis* showed high resistance to chlorpyrifos by heavily-sprayed populations, whereas recently cultivated field populations had a moderate level of resistance. A similar result was reported [21], which revealed that the four field populations collected from different governorates displayed varied tolerance or resistance level to the compounds tested for carbamate and pyrethroid. It also showed variations in resistance among field populations depending on the group of insecticides applied. In another study [22], it was found that *Spodoptera littoralis* in field populations was tolerant to the OP-insecticide (chlorpyrifos) higher than laboratory strain.

Esterase is one of the enzymes which cause the detoxification of the insecticides leading to the resistance development against the insecticides. Esterase level is directly related to insecticide resistance [23]. Increased esterase activity is a major mechanism of insecticide insensitivity or even resistance in many of insect species [5, 19]. In this study, the results showed that the relative activities of Total-E, ChE and Ali-E in all–cotton leafworm field populations were higher than those of laboratory susceptible populations. There were also differences in the degree of activity between ChE and Ali-E in a susceptible- and field-populations. The Ali-E level was variable but slightly lower in field-populations than level ChE. These finding were in agreement with those of past research [14], reporting ChE and Ali-E activity higher in the resistant strain of the *Spodoptera littoralis* than that of the susceptible strain. They also reported that the ChE and Ali-E activity was less sensitive to methyl parathion in the OP resistant strain of *S. littoralis* than in the susceptible strain. It was also mentioned that ChE and Ali-E activity was less sensitive to methyl parathion in the OP resistant strain of *S. littoralis* than in the susceptible strain. Further, ChE and Ali-E activity in OP resistant strain of *S. littoralis* was approximately 75% of that in the susceptible strain. Studies [24, 25] found the esterase activities of most field populations were higher than those susceptible populations of *Spodoptera litura*. According to a study [23], resistance to OP-insecticides (profenofos) in the field population of *Spodoptera littoralis* (Boisd.) was associated with a decrease in ChE sensitivity.

In general, the statistical analysis of the obtained K_m values was higher in all field populations than in susceptible population. These values for ChE were mainly higher than those for Ali-E in field populations. The change in the K_m values of ChE and Ali-E in all populations indicates a change in the affinities of such esterases towards α-NA. The ChE affinity towards α-NA in the larval head of *S. littoralis* is higher than that of Ali-E in field populations. The results also show that the V_{max} values of ChE and Ali-E are significantly higher in all field populations of the susceptible

population. This fact indicated that the number of active sites on ChE and Ali-E of the 4th larval head homogenates were increased in the field populations. Such changes in the field populations between ChE and Ali-E may be followed by a decrease in the insect susceptibility of the organophosphorus insecticides. These results generally indicate that ChE could participate in the occurrence of the highest resistance of the field populations. Our results are strongly supported by kinetic studies of Zhu and Gao [26], which indicated that ChE from three OP-resistant strains of the *Schizaphis graminum*, had lower affinity but higher catalytic activity acetylthiocholine iodide than ChE from the OP-susceptible strain. Based on a study [20b], both the calculated K_m and V_{max} values were higher in the field strains than the susceptible strain of *Spodoptera littoralis*. These results suggest that esterase enzymes (ChE and Ali-E) in general have a major role in the tolerance of *S. littoralis* to OP- insecticides.

The experiments showed that there were differences in the sensitivity of these two enzymes (ChE and Ali-E) in the susceptible strain as well as in field-populations of chlorpyrifos. The I_{50} values for Ali-E in the susceptible- and field-populations were smaller than those for ChE, indicating that Ali-E is more sensitive to inhibition by organophosphorus compounds. However, the susceptible strain showed a higher sensitivity to inhibition of both ChE and Ali-E than all field-populations. These results generally revealed that ChE was more active in the field-populations in the presence of chlorpyrifos, indicating that ChE could be engaged in the highest resistance to chlorpyrifos in the field-populations. Thus, esterase enzymes (ChE and Ali-E) in particular have major role in *S. littoralis* tolerance to OPs insecticides. These findings agree with those of a previous study [14], reporting that ChE activity in the OP-resistant strain of *Spodoptera littoralis* (Boisd.) was less sensitive to methyl parathion than in the susceptible strain. Ismail [21] showed the I_{50} values in the field strains of *S. littoralis* were less sensitive to chlorpyrifos and thiodicarb than susceptible strain.

5. Conclusion

In this study, the susceptibility of *Spodoptera littoralis* larvae to OPs compounds appears to be limited by increased levels of some esterases (Cholinesterase and Aliesterase) as defense agent that increases insect resistance. Thus, the data obtained in this study provide helpful information for designing a more appropriate strategy for managing *S. littoralis* resistance to insecticides in the field.

Conflict of interest

None.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

Authors' contributions

The author designed and performed the idea, analyzed the data and wrote the manuscript.

Ethics approval and consent to participate

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