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Biochemical studies of Na^+,K^+-ATPase and AChE sensitivity to insecticides and fertilization against *Spodoptera littoralis* Larvae

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ABSTRACT

The interaction of Cyhalothrin and Chlorpyrifos with fertilizer (Superphosphate, Urea, and Potassium sulphate) were determined against lab and field strains of *Spodoptera littoralis* larvae. The results showed that Cyhalothrin was the more potent toxicity than Chlorpyrifos. The LC_{50} effect of the tested compounds on the *in vivo* inhibition of Na^+,K^+-ATPase and AChE from *Spodoptera littoralis*, was assayed. The interaction effect of fertilizer with Cyhalothrin and Chlorpyrifos were investigated. Results proved that Cyhalothrin and Chlorpyrifos caused more toxicity effect when pretreated with fertilizer than single treatment. The sensitivity of Na^+,K^+-ATPase and AChE activity to the two tested insecticides were measured by the I_{50} values, the I_{50} values of Cyhalothrin pretreated with Superphosphate on lab and field strains larval Na^+,K^+-ATPase are 0.50 µM and 0.64 µM respectively, while these values are 0.63 µM and 0.71 µM respectively, against lab and field strains larval AChE for Chlorpyrifos pretreated with Urea. The inhibition constant (K_i) values were determined for Na^+,K^+-ATPase and AChE inhibitor, values of K_i in the case of Cyhalothrin pretreated with Superphosphate on lab and field strains larval Na^+,K^+-ATPase are 30 µM and 44 µM respectively, while these values are 42 µM and 56 µM respectively, against lab and field strains larval AChE for Chlorpyrifos pretreated with Urea. Generally, fertilizer pretreated with Cyhalothrin and Chlorpyrifos will produce a new trend so as increase toxicity of the insecticides, enhance the role of beneficial insects. The results of the present study may add some forward steps to use as alternative to conventional insecticides especially against this insect. So, the tested compounds can be involved in important steps necessary for successful IPM programmes applied against *S. littoralis.*

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INTRODUCTION

The cotton leafworm, *Spodoptera littoralis* is considered as one of the most serious and destructive phytophagous Lepidopterous insect-pests in Egypt, not only for cotton plants but also for more than 70 cultivated field crops and vegetables [7,2]. The indiscriminating use of insecticides has caused a number of ecological, economical and social hazardous ecological agro-ecosystems around the world including Egypt. Besides, the resistance to pesticides appeared in several insect pests. Therefore, the results from this investigation could be attributed to the possibly right use of each fertilization as control mean in the Integrated Pest Management (IPM) of the *Spodoptera littoralis* to avoid the increasing use of conventional insecticides and reduce the occurring environmental pollution.

Fertilization might be good tool to produce a profitable cotton crop that competes with weeds and able to out-grow and overcome the possible occurrence of disease and insect damage, also corrected deficiencies of certain required nutrients in large amounts (macro-elements) and \ or required in trace amounts (micro-elements). [16,14].

The present investigation aimed to study the efficiency of insecticides, (Cyhalothrin and Chlorpyrifos) either alone or in their combination with fertilizer (Superphosphate, Potassium sulphate and Urea) on *Spodoptera* larvae. Also to study a two target in the insect to the knowleage about insecticide susceptibility, describe the development of a biochemical assay system for measuring the sensitivity of Na^+,K^+-ATPase and AChE to Cyhalothrin and Chlorpyrifos respectively, and also provide enzyme kinetic data for in field strain and compared with data obtained of lab strain.

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MATERIALS AND METHODS

1. Test insect:

The susceptible laboratory strain of cotton leafworm, *Spodoptera littoralis* was provided from centeral lab of pesticides. Agricultural Research Center (ARC) Cairo, Egypt which was reared for several years on artificial diet under standard laboratory conditions of 27 ± 2 ^{o}C and 65-70% RH.

Field strain was obtained by the collection of the egg masses from cotton fields at Abeis area Alex. Proviance Egypt, the eggs were allowed to hatch larvae, chosen for bioassays, the 2nd , 3rd and 4th instar larvae and the 2nd larval instar chosen for biochemical assessments.

2. Chemicals and test insecticides:

Cyhalothrin provided as technical grade Pyrethroids insecticide from U.S.A. Environmental Protection Agency (EPA), U.S.A. Ouabain is a cardiac glycoside which specifically inhibits the Na^{+},K^{+}-ATPase (Mcllwain, 1963). A pure sample was obtained from Sigma Chem., Co. ST. loius. Dursban (Chlorpyrifos 48% EC) Organophosphate insecticide was obtained from Dow AngroSciences Co., (Dow England).

Fertilization, Urea fertilizer (46.5% N); Superphosphate fertilizer (15.5% P_2O_5), and Potassium sulphate fertilizer (48% K_2O), were supplied by Easterna Co. for Agriculture Development (Easterna, Egypt).

3. Bioassay tests:

3.1. Toxicity of the tested insecticides against S. littoralis:

Cyhalothrin and Chlorpyrifos were bioassayed against the larvae of *S. littoralis*. The castor leaves were dipped in different concentrations of the tested insecticide, Cyhalothrin concentrations were prepared in pure acetone, while Chlorpyrifos concentrations were prepared in distilled water. Treated and control plants were air-dried for 3 hrs, the treated leaves were placed in clean glass container at the laboratory conditions of 27±2 ^{o}C and 65-70 % RH, ten larvae (Lab and Field strains) were used for each test with three replicate at least. Number of alive and dead larvae per replicate was counted 24, 48, and 72hr, after treatment, concentrations–mortality percentages were calculated and corrected for natural mortality according to Abbott equation [1]. LC_{50} values were calculated by using the of probit-analysis method of Finney [6].

3.2. Toxicity of tested insecticides in presence of fertilization against S. littoralis larvae:

Larvae of *S. littoralis* were starved for 6hrs before exposed test the selected larvae was bioassayed against fertilization, (Urea, Superphosphate, and Potassium sulphate) using three replicates for each concentration with ten larvae in each replicate.

Disc dipping technique was used since it has been proved to be the most common procedure for assessing mineral fertilization. Each castor leaves disc (2cm^2) was dipped into the suspension of tested mineral fertilization for 10s. Tested concentration were prepared in glass distilled water (GDW) [21] disc were held vertically to allow excess solution to drip off and places on a rack to dry for at last 2 hr. Treated discs were offered to starved larvae (on disc per cup) and left under constant conditions (27 ± 2 ^{o}C and 65-70 % RH). There after survivors were transferred with fresh castor oil plant leaves to clean cups and kept under the same conditions.

S. littoralis larvae (lab and field strains) were treated with solution of Cyhalothrin and Chlorpyrifos at different concentrations before 24, 48, and 72 hrs of feeding on discs of castor oil leaves discs treated with tested fertilization, joint action experiments have two controls. Larvae of the first control were allowed to fed castor oil leaf discs treated with tested fertilization alone, while larvae of the second control were fed with distilled water. Mortality counted and recorded daily for 3 days. Percentage of mortality were calculated according to Abbott [1] and subjected to probit analysis [6].

4. Biochemical studies:

4.1. AChE preparation and activity assay:

AChE was prepared from *Spodoptera littoralis* 2nd instar larvae was homogenized in Tris-HCl buffer (pH 7.4) at 30 larvae / 30 ml buffer, with polytron mixer (at 50 % power for 50 sec.), then subjected to low speed centrifuged at 5,000 rpm for 15 min at

4^{o}C. The resulting supernatant was centrifuged at 15,000 rpm for 20 min at 4 ^{o}C. The supernatant centrifuged at 25,000 rpm for 1 hr at 4 ^{o}C. Pellets were resuspended in 1 ml of Tris-HCl buffer (pH 7.4) and stored at (-20 ^{o}C) for used as enzyme source, the AChE activity measurements were done according to method reported by Ellman *et al.* [5].

4.2. Na^{+},K^{+}-ATPase preparation and activity assay:

Na^{+},K^{+}-ATPase was prepared from *Spodoptera littoralis.* 2nd instar larvae was homogenized in a solution of 0.32 M sucrose, 1 mM EDTA and 40 mM Tris-HCl buffer (pH 7.4). The homogenate was filtered through two

layers of cheese cloth. Mitochondrial. ATPase was prepared according to the method reported by Koch [10], by differential centrifugation of the homogenate at 8000 Xg for 10 min. The supernatant was then centrifuged at 20000 Xg for 30 min. The formed pellets were then suspended in the same buffer and stored at (-20 ^{o}C) for use.

ATPase activity was measured according to the method reported by koch [10] with slight modification by Morshedy [15]. Inorganic Phosphate (Pi) was determined according to the method, described by Taussky and Shorr, [20]. The activity of Mg^{2+}-ATPase was measured after the addition of 1 mM Ouabain, whereas the activity of Na^{+},K^{+}-ATPase was calculated as the difference between the total ATPase and Mg^{2+}-ATPase activities.

The protein content in prepared homogenates of *S. littoralis* was assayed spectrophotometrically by the method of Lowery *et al.* [12] at λ 750 nm using Bovine Serum Albumin (BSA) as a standard protein.

2.3. In vivo inhibition of AChE and Na^{+},K^{+}-ATPase activity:

In the inhibition studies, of AChE and Na^{+},K^{+}-ATPase activity, 10 µl of the enzyme preparation was incubated with of the inhibitor for 30 min, the enzyme- inhibitor mixture was used to measure the remaining activity. The percent inhibition was calculated using the following formula:- $\%\text{Inhibition} = \frac{V-Vi \times 100}{V}$

Where:- (V) is the specific activity without inhibitor
(Vi) is the specific activity in presence of inhibitor.

2.4. In vitro inhibition and kinetics of AChE and Na^{+},K^{+}-ATPase activity:

The inhibition of Na^{+},K^{+}-ATPase activity was determined in 2nd instar larvae using the LC_{50} values of the two tested compounds (Chlorpyrifos and Cyhalothrin). The inhibitor of AChE and Na^{+},K^{+}-ATPase were evaluated to determine enzyme kinetic parameters. The method of Dixon-plots by plotting 1/V versus concentrations of the inhibitor at two concentrations of the substrate, ATP (the substrate of ATPase) concentrations were 3.0 and 5.0 mM.

Estimation of I_{50} value carried out by preincubating the enzyme with the inhibitor for 30 min, using the following concentrations 0.1, 1, 5, 10, 50 and 100 µM. K_i (the inhibition constant) values for each inhibitor were estimated from Dixon-plot.

Michaelis-Menten Kinetics (K_m and V_{max}) values were calculated by a linear regression of 6 points on each Lineweaver and Burk plot.

RESULTS AND DISCUSSION

Toxicity of tested insecticides pretreated with the tested fertilization against different *Spodoptera* larval instars:

The results of the toxicity of the Cyhalothrin and Chlorpyrifos in terms of LC_{50} are given in Table (1) for 2nd , 3rd and 4th instar larvae of *S. littoralis*. LC_{50} values were 0.039 and 0.055 ppm for Cyhalothrin and Chlorpyrifos respectively against 2nd instar larvae of *Spodoptera* lab strain, for field strains LC_{50} values were 0.055 and 0.069 ppm for two tested insecticides respectively, while LC_{50} values were 0.059 and 0.067 ppm for Cyhalothrin and Chlorpyrifos respectively against 3nd instar larvae of *Spodoptera* lab strain, for field strains LC_{50} values were 0.073 and 0.079 ppm for two tested insecticides respectively, the LC_{50} values were 0.075 and 0.078 ppm for Cyhalothrin and Chlorpyrifos respectively against 4th instar larvae of *Spodoptera* lab strain, for field strains LC_{50} values were 0.086 and 0.088 ppm for two tested insecticides respectively. According to the LC_{50} values, it is quite clear that the susceptibility of *Spodoptera* larvae to Cyhalothrin when pretreated with Superphosphate, and lab strain of *Spodoptera* larvae is more susceptible to Cyhalothrin when pretreated with Superphosphate in comparison to the field strain, general pattern was observed for the three instars, where the toxicity is decreased by the increasing in the insect instars. The second instar is more susceptible than the third and fourth instars respectively, While the lab strain of *Spodoptera* larvae is more susceptible to Chlorpyrifos when pretreated with Urea in comparison to the field strain. These results are in agreement with many investigators, [8,4,16,11,13,14].

Toxicity of tested insecticides alone or pretreated with tested fertilization against S. littoralis larvae:

Data in Table (2) show the LC_{50} values of Cyhalothrin are 0.063, and 0.044 ppm after 24, and 48 hr for 2nd instar larvae of lab *S. littoralis* strain respectively, while the LC_{50} values are 0.070, and 0.065 ppm against field *Spodoptera* strain respectively. The LC_{50} values of Chlorpyrifos are 0.071, and 0.053 ppm after 24, and 48 hr against 2nd instar larvae of lab strain respectively, while the LC_{50} values are 0.084, and 0.070 ppm against field strain respectively. The interaction of Cyhalothrin and Chlorpyrifos with tested fertilization against lab and field strains of *Spodoptera* larvae were studied larvae were allowed to feed on castor oil discs treated with LC_{50} of the tested fertilization.

Table 1: LC_{50} values of tested insecticides pretreated with the tested fertilizer against different *Spodoptera* larval instars.

Compounds	LC_{50} (ppm)					
	2nd		3rd		4th	
	Lab strain	Field strain	Lab strain	Field strain	Lab strain	Field strain
Cyhalothrin	0.039	0.055	0.059	0.073	0.075	0.086
Superphosphate +Cyhalothrin	0.020	0.044	0.044	0.060	0.062	0.074
Potassium sulphate+ Cyhalothrin	0.025	0.047	0.048	0.066	0.067	0.079
Urea + Cyhalothrin	0.034	0.050	0.054	0.070	0.073	0.083
Chlorpyrifos	0.055	0.069	0.067	0.079	0.078	0.088
Urea + Chlorpyrifos	0.042	0.054	0.053	0.064	0.063	0.070
Superphosphate+ Chlorpyrifos	0.048	0.057	0.059	0.068	0.070	0.079
Potassium sulphate+ Chlorpyrifos	0.053	0.064	0.063	0.074	0.075	0.084

The LC_{50} values of Cyhalothrin and Chlorpyrifos pretreated with the tested fertilization (Urea, Superphosphate, and Potassium sulphate) on lab and filed strains of *Spodoptera* larvae are presented in Table (2). The LC_{50} values of Cyhalothrin and Chlorpyrifos when pretreated with tested fertilization were higher than LC_{50} Cyhalothrin and Chlorpyrifos alone in lab or filed *Spodoptera* strains.

The enhancement of toxicity is calculated as a Potentiation factor (P.f.) Table (2). Potentiation factor (P.f.) values for Cyhalothrin are 2.1, 1.6, and 1.1 respectively, when pretreated with Superphosphate, Potassium sulphate, and Urea after 24 hr treatment for lab strain, while the P.f. values are 1.7, 1.5, and 1.1 respectively, when pretreated with tested fertilization 24hr treatment for field strain, the P.f. values are 3.1, 2.0, and 1.3 respectively, when pretreated with tested fertilization after 48hr treatment for lab strain, the P.f. values are 2.0, 1.6, and 1.2 respectively, when pretreated with tested fertilization 48hr treatment for field strain. Also when pretreated with Urea, Superphosphate, and Potassium sulphate the P.f. values are 1.8, 1.3, and 1.1 for Chlorpyrifos respectively, after 24hr for lab strain, while the P.f. values are 1.6, 1.2, and 1.1 respectively, after 24 hr treatment for field strain, the P.f. values are 2.2, 1.5, and 1.2 respectively, when pretreated with tested fertilization after 48 hr treatment for lab strain, the P.f. values are 1.6, 1.2, and 1.1 respectively, when pretreated with tested fertilization 48 hr treatment for field strain.

It is clear that the LC_{50} values concentrations of tested fertilization enhancement the toxicity of the tested insecticides on *S. littoralis* larvae. Cyhalothrin when pretreated with Superphosphate were the most toxic treatments than pretreated with Potassium sulphate, and Urea respectively, while Chlorpyrifos when pretreated with Urea were the most toxic treatments than pretreated with Superphosphate, and Potassium sulphate respectively, these results are in agreement with which found by many [7,19,17].

Table 2: Comparative toxicities of tested insecticides alone or pretreated with tested fertilizer on *Spodoptera* larvae.

Compounds	LC_{50} (ppm)							
	24hr				48hr			
	Lab strain	P.f.*	Field strain	P.f.	Lab strain	P.f.	Field strain	P.f.
Cyhalothrin	0.063		0.070		0.044		0.065	
Superphosphate +Cyhalothrin	0.030	2.1	0.042	1.7	0.014	3.1	0.033	2.0
Potassium sulphate+ Cyhalothrin	0.040	1.6	0.048	1.5	0.022	2.0	0.042	1.6
Urea + Cyhalothrin	0.056	1.1	0.066	1.1	0.035	1.3	0.055	1.2
Chlorpyrifos	0.071		0.084		0.053		0.070	
Superphosphate+Chlorpyrifos	0.067	1.1	0.075	1.1	0.046	1.2	0.065	1.1
Potassium sulphate+ Chlorpyrifos	0.055	1.3	0.068	1.2	0.035	1.5	0.057	1.2
Urea + Chlorpyrifos	0.040	1.8	0.053	1.6	0.024	2.2	0.044	1.6

*Potentiation factor (P.f.) = LC_{50} tested insecticide alone / LC_{50} tested insecticide + tested fertilizer

In general the susceptibility of *Spodoptera* larvae to Cyhalothrin increase when treatment after Superphosphate, the observation that Cyhalothrin had the lowest effect when applied alone but it was the best when mixed with Superphosphate. The Superphosphate+Cyhalothrin, and Urea+Chlorpyrifos caused more toxic than effect single treatment. So may be these effect of tested fertilization in mixture and these are a good control of Lepidopterous larvae, it could be concluded that tested insecticide enhanced the toxicity effect of tested fertilization. Based on P.f. values, the lab strain of *Spodoptera* larvae is more susceptible comparison to the field

strain. Generally, efficacy of tested fertilization have a very good additive toxicity for Pyrethroids (Cyhalothrin), and Organophosphate (Chlorpyrifos) either in lab or field *Spodoptera* strains.

In vivo inhibition of S. Littoralis Na^+,K^+-ATPase and AChE activity:

The *in vivo* inhibitory effect of the LC_{50} values of tested insecticides against to the *Spodoptera* 2nd instar lab and field strains larval Na^+,K^+-ATPase and AChE are shown in the data given in Table (3 and 4). The data declared that Cyhalothrin and Chlorpyrifos exhibited the percentages of reduction of Na^+,K^+-ATPase and AChE activity as values were 80.1 and 77.4 % respectively, for lab strain, while values was 77.3 and 70.7 % respectively, for field strain.

Data in Table (3 and 4) summarize the interaction of tested fertilization on the inhibitory effect of Cyhalothrin and Chlorpyrifos on the activity of Na^+,K^+-ATPase and AChE. The resultes proved that pretreated of tested fertilization with Cyhalothrin induce increase the inhibition of enzyme activity. The inhibition of Na^+,K^+-ATPase by Cyhalothrin alone were 80.1 and 77.3 % for lab and field strains respectively, while the inhibition increased to be 90.5 and 88.6 % for lab and field strains respectively when pretreated Superphosphate, the inhibition of AChE by Chlorpyrifos alone were 77.4 and 70.7 % for lab and field strains respectively, while the inhibition increased to be 86.6 and 83.5 % for lab and field strains respectively when pretreated with Urea. This results agreement with Smagghe, and Degheele, [19] Casida and Quistad [3] and Saleem, [17].

Table 3: *In vivo* inhibition of *Spodoptera* larvae 2nd instar Na^+,K^+-ATPase activity by tested tested fertilizer (LC_{50}).

Compounds	%Inhibition	
	Lab strain	Field strain
Cyhalothrin	80.1	77.3
Superphosphate +Cyhalothrin	90.5	88.6
Potassium sulphate+ Cyhalothrin	85.3	83.8
Urea + Cyhalothrin	83.2	79.1

Table 4: *In vivo* inhibition of *Spodoptera* larvae 2nd instar AChE activity by tested fertilizer (LC_{50}).

Compounds	%Inhibition	
	Lab strain	Field strain
Chlorpyrifos	77.4	70.7
Urea + Chlorpyrifos	86.6	83.5
Superphosphate + Chlorpyrifos	80.5	77.8
Potassium sulphate+ Chlorpyrifos	79.2	72.4

The in vitro inhibition of S. Littoralis Na^+,K^+-ATPase and AChE activity:

Table (5) The *in vitro* interaction of Cyhalothrin and Chlorpyrifos on Na^+,K^+-ATPase and AChE activity of *Spodoptera* 2nd instar respectively. The I_{50} values of Cyhalothrin for lab and field strains larval Na^+,K^+-ATPase are 0.73 and 0.80 µM respectively, these values of Chlorpyrifos for lab and field strains larval AChE are 0.81 and 0.88 µM respectively. On the other hand the K_i values of Cyhalothrin for lab and field strains larval Na^+,K^+-ATPase are 51 and 60 µM respectively, the values of These values of Chlorpyrifos for lab and field strains larval AChE are 63 and 75 µM respectively.

Generally, it was noticed that pretreated of tested fertilizer with the tested insecticides clearly decreases values of I_{50} and K_i the lowest recorded I_{50} and K_i values of the mixture between fertilizer and insecticide reflect that the fertilizer may active the insecticide to inhibit the enzyme, in the other words, adding the fertilizer to the insecticide increased its inhibition potency. We study the *in vitro* biochemical interaction of them with the Cyhalothrin and Chlorpyrifos *in vitro* effects.

Table 5: *In vitro* inhibition of *Spodoptera* larvae Na^+,K^+-ATPase and AChE activity by tested insecticides.

Compounds	I_{50} (µM)		K_i (µM)	
	Lab. strain	Field strain	Lab. strain	Field strain
Cyhalothrin	0.73	0.80	51	60
Chlorpyrifos	0.81	0.88	63	75
Superphosphate +Cyhalothrin	0.50	0.64	30	44
Urea + Chlorpyrifos	0.63	0.71	42	56

To characterize more details about the *in vitro* inhibition of Na^+,K^+-ATPase and AChE by the inhibitor, the I_{50} and K_i values of each inhibitor were estimated from the graphical method of Dixon and Weeb, (Table 4). The obtained data proved that compounds competitive inhibition of Na^+,K^+-ATPase activity and K_i values were 30

and 44 µM for lab and field strains in the case of Cyhalothrin pretreated with Superphosphate, while the obtained data proved that compounds competitive inhibition of AChE activity and K_i values were 42 and 56µM for lab and field strains respectively in the case of Chlorpyrifos pretreated with Urea.

It is concluded from the present results that the tested Pyrethroids and Organophosphate are potentially potent for control of *S. littoralis* however, with tested compounds, such as Fertilization currently in use, *S. littoralis* could be successfully included in the pest management programs, also, it is quite clear that when certain pairs of drugs or insecticides are administered together, the effects may be greater or less than might be expected from the sum of the activities of the components when administered separately. The phenomena involved, included under the term "synergism" "potentiation" and "antagonism", are becoming increasingly important in, for example, practical insect control and mammalian toxicology.

General, it could be concluded that the use of Pyrethroids (Cyhalothrin) and Organophosphate (Chlorpyrifos) and their mixtures with Fertilization (Superphosphate, Urea, and Potassium sulphate) instead of conventional hazardous insecticides; and these my reduce the environmental pollution and hazard effects on human health. tested compounds may play an important role in future insect pest management programs.

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