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Biochemical Mechanisms and Cross Resistance Patterns of Chlorpyrifos Resistance in a Laboratory-Selected Strain of Diamondback Moth, *Plutella xylostella* (Lepidoptera: Plutellidae)

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ABSTRACT

Diamondback moth (*Plutella xylostella*) is a serious pest of cruciferous vegetables worldwide. In Iran, it is commonly controlled by using chlorpyrifos. Due to a range of biochemical and behavioral features, this pest can rapidly develop resistance to many insecticides from different groups. To achieve a better resistance management plan, a chlorpyrifos resistant strain of *P. xylostella* was selected under laboratory conditions and its cross resistance to five other insecticides and resistance characteristics were investigated. After 15 generations of selection, the selected strain (CLRS) developed 39.61-fold higher resistance to chlorpyrifos in comparison with susceptible strain (AL). CLRS exhibited 19.62-, 17.84-, 3.43- and 3.33-fold cross resistance to hexaflumuron, indoxacarb, thiodicarb and flubendiamide, respectively, but showed no cross resistance to abamectin. Synergism and biochemical studies suggested potential involvement of Esterase (EST) in CLRS. However, no difference was seen for Glutahion-S-Transferase (GSTs) and Mixed Function Oxidase (MFO) in CLRS and AL strains. To determine the role of AcetylCholinEsterase (AChE) insensitivity in resistance mechanism, Kinetic parameters (K_m and V_{max}) and inhibitory effect of chlorpyrifos-oxon on this enzyme were evaluated. Affinities and hydrolyzing efficiencies of AChE in CLRS were higher than AL. This enzyme in CLRS was also less sensitive to inhibition by chlorpyrifos-oxon. Results indicated that chlorpyrifos resistance exhibited cross resistance to other insecticides from different classes and enhanced EST activity and AChE insensitivity were probably the main factors in chlorpyrifos resistance. These results can help the users of insecticides and can delay the resistance development of *P. xylostella*.

Keywords: Acetylcholine esterase, Brassicaceae, Insecticide resistance, Resistance management plan, Synergism.

INTRODUCTION

Brassicaceae family is a large and diverse plant group that includes economically important crops such as mustard, cabbage, cauliflower, broccoli, rapeseed, turnip, radish and Chinese cabbage. These crops are the most prominent groups of vegetables in Asian countries and their successful production is restricted by some destructive insect pests. The diamondback moth (*Plutella xylostella* (L.): Lepidoptera: Plutellidae) is a major and widely distributed pest of cruciferous crops (Talekar and Shelton, 1993; Salinas, 1977; Sarfraz and Keddie, 2005; Golizadeh *et al*., 2009). Most damage is created by the larvae tunneling into the head/foliage parts of host plants

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(Troczka *et al*., 2017). Insecticide application is still one of the major pest control programs. In Iran, total pesticide application by farmers on cabbage productions could reach 15-20 times in one season (A. Soleymanzade, 2018, unpublished data). Excluding crop loss, the cost of *P. xylostella* control is estimated to be US $ 1.4 billion per year. However, due to its short generation time, high fecundity and genetic elasticity, the pest shows high levels of resistance to a broad range of insecticides (Sarfraz and Keddie, 2005; Furlong *et al*., 2012). To date, this pest has developed resistance to 95 different insecticides of more than ten classes of modes of action (Steinbach *et al.*, 2017). *P. xylostella* has also developed cross-resistance to many different groups of insecticides (Shelton *et al*., 2000).

Chlorpyrifos, an organophosphate insecticide, is effective against many insect pests (Wang *et al*., 2010; Askari Saryazdi *et al*., 2015). This insecticide is used in protecting a number of main agricultural crops. Also, it is an officially recommended insecticide in diamondback moth control (Asare-Bediako *et al*., 2010). At present, the control of *P. xylostella* in Iran is primarily dependent on the application of some common insecticides, like deltamethrin, chlorpyridos, hexaflumuron and indoxacarb (A. Soleymanzade, 2018, unpublished data). Previous studies have reported high degree of resistance to organophosphate, specially chlorpyrifos, in different diamondback moth populations (Hama, 1990; Odhiambo *et al*., 2010; Gong *et al*., 2013; Agboyi *et al*., 2016).

Artificial selection of resistant strains is important because it allows the determination of resistance mechanisms and, consequently, the risk estimate of resistance development. This information is highly significant in Integrated Pest Management (IPM) strategies (Liu *et al*., 2015).

Previous studies have proven that resistance to organophosphate insecticides is due to increased detoxification by some metabolic enzymes (Gong *et al*., 2013; Askari Saryazdi *et al*., 2015; Alout *et al*., 2016) and reduced sensitivity of acetylcholine esterase (Zibaee *et al*., 2017). Enzyme inhibitors such as PiperonylBotoxide (PBO, mixed function oxidase inhibitor), TriPhenyl Phosphate (TPP, carboxyesterase inhibitor), DiEthyl Maleate (DEM, gluthation-S-transferase inhibitor) and S,S,S-tributylphosphorotrithioate (DEF, esterases inhibitor) could affect resistance development and can be applied not only as an alternative to improve control, but also as a tool for elucidating resistance mechanisms (Picollo *et al*., 2000; Kang *et al*., 2006).

It is necessary to study biochemical mechanisms and patterns of resistance to chlorpyrifos in *P. xylostella* due to its vast application in the fields against different pests of cabbage crops. Although correlations between pesticide resistance and enzyme activity have been widely studied by many researchers, there are a few studies on resistance patterns of chlorpyrifos in *P. xylostella* populations. To the best of our knowledge, characterization of AChE in this pest had not been studied and this was the first evaluation in this regard.

This study was conducted to check whether detoxification enzymes were involved in chlorpyrifos resistance in *P. xylostella*. These aims were addressed by studying synergistic effects of some inhibitors such as PBO, DEM, TPP, DEF and by evaluating enzyme activities. Cross-resistance to some other insecticides was also estimated. Such investigations can help to reduce the risk of chlorpyrifos resistance development in *P. xylostella* population.

MATERIALS AND METHODS

Insecticides and Chemicals

The insecticides evaluated in this study are listed in Table 1. Sodium dodecyl sulfate (SDS), Tween 80 and *a*-naphtol were purchased from Merck (Darmstadt, Germany). Hydrogen peroxide, 1-Chloro-2,

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Table 1. List of used insecticides with their trade names, producers, and IRAC classification.

Compound	Trade name	Manufacturer	Group	IRAC classification
Abamectin	Vertimec®, 1.8 EC	Partonar, Iran	Avermectin	Group 6
Chlorpyrifos	Dursban®, 40.8 EC	Ariashimi, Iran	Organophosphate	Group 1B
Flubendiamide	Takumi®, 20 % WG	Nihon Nohgaku, Japan	Diamid	Group 28
Hexaflumuron	Consult®, 10 % EC	Dow Agrosciences, UK	Benzoylphenyl urea	Group 15
Indoxacarb	Avanut®, 150 SC	DuPont, France	Oxadiazines	Group 22A
Thiodicarb	Larvin®, 80 % DF	Moshkfam, Iran	Carbamates	Group 1A

4-DiNitroBenzene (CDNB), *a*-Naphthyl Acetate (*a*-NA), S,S,S-tributylphosphorotrithioate (DEF), DiEthyl Maleate (DEM), fast blue RR salt, reduced Glutathione (GSH), PiperonylBotoxide (PBO), 3,3',5,5'-TetraMethylBenZidine (TMBZ), TriPhenyl Phosphate (TPP), cytochrome c, Bovine Serum Albumin (BSA), commassie brilliant blue G-250, 5,5′-DiThiobis(2-NitroBenzoic acid) (DTNB) and AcetylthioCholine Iodide (AChI) were purchased from Sigma Aldrich (Taufkirchen, Germany). Chlorpyrifos-oxon was obtained from laboratories of Dr. Ehrenstorfer.

Insects

The susceptible strain (AL) of diamondback moth was collected from cabbage plants (*Brassica* oleracea) in a field located in Ardabil Province (38° 12′ 509″ N, 48° 39′ 378″) in May 2016, where insecticides had not been applied for 4-5 years. This strain was reared in the laboratory under insecticide-free conditions and its high susceptibility to some insecticides was proved. The resistant strain (Chlorpyrifos Resistant Strain, CLRS) was selected by chlorpyrifos for 15 generations from this susceptible strain and in each generation at least 300 larvae were used. Third-instar larvae of *P. xylostella* were exposed to cabbage leaves with chlorpyrifos for 48 h. Then, surviving larvae were treated with fresh cabbage leaves. Based on former bioassays, LC_{70} value of chlorpyrifos was applied to select each generation. The larvae were reared and allowed to pupate under greenhouse conditions [25±1ºC, 60-70% Relative Humidity (RH) and photoperiod of 16: 8 (L: D)]. Adults were fed on 10% honey-water solution and allowed to lay eggs on potted radish seedlings (*Raphanus sativus* L.) in wooden framed cages (50×40×30 cm). These potted radish seedlings were replaced every one or two days and then were transferred to greenhouse where the eggs were hatched. Finally, second instar larvae were transferred to larvae cages. After 2 or 3 generations of selection, toxicity of chlorpyrifos on the third instar larvae of CLRS strain was determined.

Bioassay

The toxicity of chlorpyrifos on the third instar larvae of *P. xylostella* was evaluated using a leaf dip method suggested by Shelton *et al*. (1993). Leaf discs of cabbage plant (5.5 cm in diameter) were dipped in five concentrations of chlorpyrifos for 20 seconds and allowed to dry at 25-27°C for 1-2 hours. Then, leaf discs, each with 20 third-instar larvae, were transferred into a plastic Petri dish (6 cm in diameter). For each concentration, three replicates with a total of 60 individuals were selected. Controls were treated by the same method with distilled water and Tween-80. Larvae were scored for mortality after 48 hours and were counted as dead when having no response to fine-haired brush.

Synergism Test

Before synergism tests, toxicity was assessed with a range of synergist

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concentrations to choose a maximum sub-lethal dose with no mortality effect on the larvae. Synergists were prepared in acetone. Maximum sub-lethal doses for DEF, DEM, PBO and TPP were 150, 500, 300 and 90 mg L^{-1}, respectively. Synergism tests were performed similar to larvae bioassays, except that the third instar larvae were topically treated with 1 µL synergist solution on dorsal thoracic segments 1 h before they were fed with chlorpyrifos treated leaves.

Cross Resistance Test

Cross-resistance between chlorpyrifos and five other insecticides (abamectin, flubendiamide, hexaflumuron, indoxacarb, and thiodicarb) was evaluated on CLRS and AL strains of the pest. Toxicities of these insecticides were estimated on the third instar larvae using leaf-dipping method as described in previous sections.

Enzyme Assay

Protein Assay

The protein content of the enzyme solution was measured by Bradford method using BSA as standard.

Enzyme Preparation

Sixty third instar larvae (20 larvae/replication) were homogenized in ice-cold buffer and centrifuged at 4°C and 10,000 rpm for 20 minutes and the supernatant was collected and used as the enzyme source. The buffers used for enzyme preparation were 0.02M sodium phosphate (pH 7 containing 0.3% Triton-X 100) for Esterase (EST), 0.1M sodium phosphate (pH 6.5) for Gluthation-S-transferase (GSTs), 0.625M potassium phosphate (pH 7.2) for Mixed Function Oxidase (MFO) and 0.1M sodium phosphate (pH 7 containing 1% Triton-X 100) for AcetylCholine Esterase (AChE).

EST Activity Assay

EST activity was investigated using *a*-NA as substrate (Van Asperen, 1962). Twenty µL enzyme and 200 µL *a*-NA solution (containing 100 µL 30 mM *a*-NA dissolved in 10 mL 0.02 M sodium phosphate buffer, pH 7.2) were mixed and then incubated at 27°C for 15 minutes. Then, 50 µL fast blue RR salt solution (150 mg fast blue RR salt dissolved in 15 mL distilled water and 35 ml 5% SDS) was added to terminate the reaction. In this test, enzyme –free phosphate buffer was used as control. The plates were maintained at 25-27°C for 5 minutes; then their absorbance was measured at 450 nm. A standard curve of *a*-naphtol was constructed to measure *a*-naphtol produced during esterase assay.

GSTs Activity Assay

GSTs activity test was performed according to Habig *et al*. (1974) method with slight modifications using CDNB and reduced GSH as substrates. For this assay, 10 µL enzyme was added to the wells of a microplate reader and then 10 µL 63 mM CDNB and 190 µL 10 mM reduced GSH were added. Non-enzymatic reactions of CDNB and reduced GSH without homogenate with buffer only were served as blanks. Then, changes in absorbance values were measured at 30 seconds intervals at 340 nm and 27°C for 5 minutes. The enzyme activity was assessed based on the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB at 340 nm.

MFO Titration Assay

The method described by Brogdon *et al*. (1997) was adopted for measuring the total amount of heme-containing proteins. This is a simple method for titration of heme bound in samples. The assay measures the heme content, which is

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mainly associated with cytochrome P450 in non-blood feeding insects. For this test, the reaction mixture in each well of microplate reader contained 20 µL enzyme, 80 µL 0.625M potassium phosphate buffer (pH 7.2), 200 µL TMBZ solution (0.01 g TMBZ dissolved in 5 mL methanol+15 ml 0.25M sodium acetate buffer, pH 5) and 25 µL 3% hydrogen peroxide. Wells containing potassium phosphate buffer instead of enzyme served as controls. The plates were incubated at 27°C for 30 minutes and absorbance was measured at 630 nm. A purified cytochrome C curve was used as standard.

Kinetics, Activity, and Sensitivity of AChE

This assay was performed according to Ellman *et al*. (1961) method with some modifications. Reaction mixture included 30 µL enzyme, 178 µL 0.1M sodium phosphate buffer at pH 7.8 (containing 1% Triton-X 100), 12 µL 10 mM DTNB solution (in 0.1M sodium phosphate buffer, pH 7) and 30 µL of AChI. In inhibition and sensitivity assays, the reaction mixture included the same mixture as above plus chlorpyrifos-oxon (0.1M in acetone) as AChE inhibitor. The final concentrate of acetone in the reaction mixture was always below 1%. Absorption of the product was monitored at 405 nm for 15 minutes at 25°C. The results were assessed based on the extinction coefficient of 1.36×10^4 mM^{-1} cm^{-1}.

To perform kinetic analysis of AChE and determine K_m and V_{max} values, 12 µL DTNB (10 mM) and 30 µl AChI solution (3.6-2250 µM) were first mixed. To start the reaction, 30 µL enzyme was added. Enzyme activity was determined by monitoring the reaction at 405 nm for 15 minutes at 25°C. To measure the Kinetic parameters (K_m and V_{max}), Lineweaver-Burk plot was used.

Analysis

Bioassay data were analyzed by probit analysis using SPSS v. 17. O. Synergistic ratio (SR) was calculated by the following formula: *SR= LC_{50} value of chlorpyrifos without synergist/LC_{50} value of chlorpyrifos with synergist*. Resistance Ratios (RR) were assessed as: *RR= LC_{50} value of CLRS/LC_{50} value of AL strain*. Cross-resistance Ratio (CR) was measured by dividing the LC_{50} value of each insecticide for CLRS by the same insecticide for AL. All enzyme assays were carried out in three replications and the mean values were separated by t-test.

RESULTS

Selection of Chlorpyrifos Resistance

A susceptible strain (AL) of diamondback moth was continuously selected for 7 times with chlorpyrifos during 15 generations in the laboratory. The development trend of Chlorpyrifos Resistant Strain (CLRS) is presented in Table 2. In the selection processes, the rate of resistance development for the first 6 consecutive generations was slow and equal to 6.53. However, in the following selection, the resistance was speeded up and in generation 15, the resistance ratio was equal to 39.61.

Synergism of PBO, DEF, DEM and TPP to Chlorpyrifos

The data of chlorpyrifos toxicity on the third instar larvae after pretreatment with PBO, DEF, DEM and TPP are shown in Table 3. About 2.08 synergism for DEF was produced in CLRS strain. TPP enhanced the toxicity of chlorpyrifos to CLRS by 1.41-fold. Almost, no synergistic effect was seen for DEM and PBO in CLRS.

Cross-Resistance Evaluation

Chlorpyrifos Resistant Strain (CLRS) was examined for cross-resistance to different insecticides (Table 4). According to the obtained results, CLRS showed cross-resistance to flubendiamide (3.33), thiodicarb (3.43), hexaflumuron (19.62), and indoxacarb (17.84) compared to the

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Table 2. Selection of resistance to chlorpyrifos in different generations of *Plutella xylostella.*

Generation	*n*	Slope±*SE*	LC_{50} (mg l^{-1})	x^2 (df)	RR^a
$G_{0\ (AL)}$[b]	300	2.00 ± 0.34	54.93 (46.28-66.78)	0.66 (3)	1.00
G_2	300	1.39 ± 0.24	102.42 (79.95-135.76)	1.84 (3)	1.86
G_4	300	1.48 ± 0.25	207.61 (164.77-270.10)	1.17 (3)	3.78
G_6	300	1.55 ± 0.27	358.70 (287.62-461.23)	1.78 (3)	6.53
G_8	300	1.70 ± 0.29	768.43 (628.35-966.42)	0.86 (3)	13.99
G_{10}	300	1.85 ± 0.31	1665.25 (1383.74-2056.44)	0.76 (3)	30.31
G_{13}	300	1.94 ± 0.34	2074.44 (1738.53-2536.72)	2.42 (3)	37.76
$G_{15\ (CLRS)}$[c]	300	2.07 ± 0.36	2175.78 (1843.92-2626.96)	2.11 (3)	39.61

[a] RR: Resistance Ratio, LC_{50} of the resistant strain/LC_{50} of the parental strain; [b] AL: Susceptible parental population, [c] CLRS: Chlorpyrifos Resistance Strain.

Table 3. Toxicity of chlorpyrifos with and without synergists to third instar larvae of *Plutella xylostella*.

Strain	*n*	Insecticide	LC_{50} (mg l^{-1})	Slope±*SE*	x^2 (df)	SR^a
AL	300	Chlorpyrifos	54.93 (46.28-66.78)	2.00 ± 0.34	0.66 (3)	-
	300	Chlorpyrifos + DEM	59.34 (51.18-70.39)	2.29 ± 0.39	0.54 (3)	0.93
	300	Chlorpyrifos + DEF	33.95 (28.62-41.23)	2.01 ± 0.34	1.17 (3)	1.62
	300	Chlorpyrifos + PBO	43.30 (36.58-52.49)	2.03 ± 0.34	0.74 (3)	1.26
	300	Chlorpyrifos + TPP	73.56 (63.58-86.85)	2.35 ± 0.41	2.69 (3)	0.77
CLRS	300	Chlorpyrifos	2175.78 (1843.92-2626.96)	2.07 ± 0.36	2.11 (3)	-
	300	Chlorpyrifos + DEM	2123.44 (1797.23-2568.33)	2.05 ± 0.36	3.34 (3)	1.03
	300	Chlorpyrifos + DEF	1044.35 (935.95-1186.81)	3.11 ± 0.45	4.36 (3)	2.08
	300	Chlorpyrifos + PBO	2202.16 (1873.28-2647.34)	2.12 ± 0.36	1.55 (3)	0.99
	300	Chlorpyrifos + TPP	1544.68 (1259.94-1948.34)	1.68 ± 0.28	0.73 (3)	1.41

[a] SR: Synergistic Ratio.

Table 4. Toxicity of evaluated insecticides to the AL and CLRS strains of *Plutella xylostella*.

Insecticide	Strain	*n*	LC_{50} (mg l^{-1})	Slope±*SE*	x^2 (df)	CR^a
Abamectin	AL	300	2.99 (2.30-2.05)	1.30 ± 0.22	2.19 (3)	0.78
	CLRS	300	2.28 (1.68-3.17)	1,13 ± 0.18	2.58 (3)	
Flubendiamide	AL	300	0.003 (0.003-0.005)	1.43 ± 0.25	2.48 (3)	3.33
	CLRS	300	0.01 (0.007-0.014)	1.08 ± 0.16	0.62 (3)	
Hexaflumuron	AL	300	24.75 (20.50-30.67)	1.82 ± 0.31	1.39 (3)	19.62
	CLRS	300	485.57 (462.47-507.95)	4.56 ± 0.99	0.67 (3)	
Indoxacarb	AL	300	2.20 (1.61-3.13)	1.11 ± 0.19	1.39 (3)	17.84
	CLRS	300	39.25 (27.73-57.01)	1.01 ± 0.16	0.43 (3)	
Thiodicarb	AL	300	0.07 (0.05-0.09)	1.49 ± 0.26	2.69 (3)	3.43
	CLRS	300	0.24 (0.18-0.32)	1.25 ± 0.20	2.78 (3)	

[a] CR: Cross resistance Ratio= LC_{50} of CLRS strain/LC_{50} of AL strain.

susceptible strain. CLRS exhibited no cross-resistance to abamectin.

Enzyme Activities

To determine the role of detoxification enzymes in chlorpyrifos resistance, EST, MFO, GST, and AChE were measured in both susceptible (AL) and resistant (CLRS) strains (Table 5). No differences in the activities of GST and MFO were found between AL and CLRS strains. In contrast, the activity of EST was 3.74-fold higher in CLRS strain than AL strain.

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Table 5. Activities of different enzymes groups in third instar larvae of CLRS and AL strains of *Plutella xylostella*.

Enzyme	Strain	Enzyme activity[a] (Mean±SE)	Ratio[b]
EST Activity±*SE* (μmol min^{-1} mg $protein^{-1}$)	AL	17.28 ± 0.25	3.74
	CLRS	64.63 ± 1.01	
GST Activity±*SE* (μmol min^{-1} mg $protein^{-1}$)	AL	36.08 ± 1.58	1.02
	CLRS	36.93 ± 2.33	
MFO Activity±*SE* (μmol min^{-1} mg $protein^{-1}$)	AL	0.27 ± 0.003	1.15
	CLRS	0.31 ± 0.005	

[a] Enzyme activity was showed as means±*SE* and significance was assessed by *t*-test (P< 0.05). [b] Activity ratio= Aactivity in the CLRS strain with activity in AL strain.

Kinetics, Activity, and Sensitivity of AChE

The kinetics of AChE from CLRS and AL strains were evaluated to determine the relationship between resistance and enzyme sensitivity (Table 6). The activity of AChE was significantly different between CLRS and AL strains, and in CLRS it was about 2.01-fold higher than that of AL strain. Also, in AL strain, AChE was more sensitive to chlorpyrifos-oxon inhibition compared with CLRS strain (Table 6). The inhibition rates of AChE in CLRS and AL strains were 34.08 and 68.39%, respectively, suggesting that the enzyme in CLRS strain was significantly more tolerant to chlorpyrifos.

The affinities and hydrolyzing efficiencies of AChE in CLRS and AL strains were determined based on kinetic analysis (Table 7). The results showed that CLRS AChE affinity to AChI was 1.97-fold lower than that of AL, as indicated by K_m values (Table 7), suggesting that AChE in CLRS might become insensitive. In contrast, based on V_{max} values, hydrolyzing efficiency of AChE in CLRS was 3.53 -fold higher than AL.

DISCUSSION

Chlorpyrifos is an important pesticide in IPM systems in various crops and invasive

Table 6. Acetylcholinesterase activity in CLRS and AL strains of *Plutella xylostella*.

Strain	AChE activity±SE (μmol min^{-1} mg $protein^{-1}$)				% Chlorpyrifos-oxon inhibition
	Without inhibitor	Ratio[a]	With inhibitor	Ratio	
AL	10.18 ± 0.02	1.00	3.32 ± 0.01	1.00	68.39
CLRS	20.42* ± 0.24	2.01	13.46*±0.09	4.05	34.08

[a] *Ratio= Values of CLRS AChE activity/AL AChE activity.* * Indicated that the mean from the CLRS is significantly different from that of the AL (P< 0.05) by student's *t*-test.

Table 7. Kinetic parameters of AChE from AL and CLRS strains of *Plutella xylostella*.

Strain	K_m±*SE* (μM)	Ratio [a]	V_{max}±*SE* (μmol min^{-1} mg $protein^{-1}$)	Ratio[a]
AL	18.06 ± 0.01	1	0.93 ± 0.006	1
CLRS	35.65* ± 2.94	1.97	3.28* ± 0.178	3.53

[a] *Ratio= Values of CLRS K_m or V_{max}/AL K_m or V_{max}.* * Indicated that the mean from the CLRS is significantly different from that of the AL (P<0.05) by student's *t*-test.

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pests due to its efficacy and potency (Ejaz *et al*., 2016). For evaluation of resistance risk, an insecticide susceptible strain of *P. xylostella* (AL) was continuously selected under chlorpyrifos pressure in laboratory. After 15 generations of selection, 39.61-fold higher resistance was generated. To the best of our knowledge, there is no report on this level of *P. xylostella* resistance to chlorpyrifos and our results indicated that CLRS had the capability of developing high resistance to chlorpyrifos under selection pressure, which agrees with some reported chlorpyrifos resistance monitoring studies. After laboratory selection for 25 generations, 158.58-fold (Xu *et al*., 2013) and 188-fold (Wang *et al*., 2010) higher resistances to chlorpyrifos have been reported in resistant strain of *Laodelphax striatellus* in comparison with susceptible strain. Also, after 23 generations of laboratory selection on *L. sative*, a resistant strain with resistance ratio of 40.34 was obtained (Askari Saryazdi *et al*., 2015).

Previous studies have documented that two insecticides might show cross-resistance when they had the same effect on the main detoxification enzymes (Liu *et al*., 2015). Unfortunately, there is no documented investigation on cross-resistance of chlorpyrifos with other insecticides. In the present study, moderate cross-resistance was observed between flubendiamide and chlorpyrifos in CLRS strain of *P. xylostella*. CLRS showed noticeable cross-resistance to indoxacarb and hexaflumuron. Because these three insecticides (chlorpyrifos, indoxacarb, and hexaflumuron) have different modes of action, the cross resistance should be based on some factors other than target insensitivity. Nehare *et al*. (2010) indicated that organophosphate-resistant strain of *P. xylostella* showed positive cross resistance to indoxacarb, which agrees with our findings. In our work, very low or no cross resistance with abamectin was observed. Wang *et al*. (2010) also reported that the *L. striatellus* strain resistant to chlorpyrifos had no cross-resistance to abamectin. Similarly, Askari Saryazdi *et al*. (2015) observed no cross-resistance between chlorpyrifos and abamectin in the chlorpyrifos resistant strain of *L. sative*. These results are in agreement with our findings. Thus, there should be no problem in using chlorpyrifos in alternation with abamectin, and it is recommended.

In the present study, DEF (esterase inhibitor) exhibited significant synergistic effect on chlorpyrifos in CLRS strain of *P. xylostella* and biochemical assays displayed an increase in EST activity. Using synergism/biochemical studies, some researchers have proven that EST played a key role in the hydrolysis of chlorpyrifos in the resistant insect pests. Enhanced EST activity was found in chlorpyrifos resistant strain of *Culex quinquefasciatus* (Selvi *et al*., 2007), *L. striatellus* (Wang *et al*., 2010), *Bemisia tabaci* (Zhang *et al*., 2012) and *L. sativae* (Askari Saryazdi *et al*., 2015). Based on our results, it is difficult to determine the roles of MFO and GSTs on chlorpyrifos resistance in diamondback moth. The inhibitors of these enzymes had no significant synergism on chlorpyrifos toxicity and the enzyme activities were similar in both resistant and susceptible strains. Xu *et al*. (2013) showed that GST was not a major factor in chlorpyrifos resistance in *L. striatellus;* but Yang *et al*. (2009) reported that EST and GST played major roles in conferring malathion resistance in *Locusta migratoria* (Meyen).

Resistance to some organophosphates is conferred by increased activity of target enzyme AChE. This phenomenon has been reported for some pests such as *Schizaphis graminum* (Zhu and Gao, 1999) and *Bactrocera dorsalis* Hendel (Shen *et al*., 2012). The current study found that the CLRS strain had higher AChE activity (2.01-fold) compared with AL strain and synergism data of TPP well matched with these findings.

Kinetic analysis indicated that AChE from CLRS was significantly different from that of AL. Such differences were reflected in reduced sensitivity to chlorpyrifos-oxon inhibitor and the decreased affinity to AChI.

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The K_m value in CLRS strain was significantly higher indicating that AChE insensitivity was probably involved in the resistance of *P. xylostella* to chlorpyrifos. The V_{max} value of CLRS was also higher than AL strain, suggesting that overexpression or increased activity of AChE could also contribute to the observed resistance. Some other studies have also reported similar results in other chlorpyrifos resistant pests including *Tetranychus urticae* Koch. (Zamani *et al*., 2014) and *L. striatellus* (Wang *et al*., 2010). Further studies are needed to reveal the structural modifications of AChE in CLRS strain.

In conclusion, this study revealed the potential of high chlorpyrifos resistance development in *P. xylostella* and cross-resistance patterns to five other insecticides. Based on synergism and biochemical mechanism studies in Chlorpyrifos selected Resistant Strain (CLRS) and susceptible strain (AL), different detoxification enzyme activities were evaluated. Chlorpyrifos resistance in CLRS strain was partly due to EST-mediated metabolic detoxification as evidenced by significant synergistic effect of DEF on the toxicity of the chlorpyrifos and significant increase in EST activity. In addition, increased activity and reduced sensitivity of AChE also proved chlorpyrifos resistance in CLRS strain. Increased K_m value and less sensitivity to inhibition by chlorpyrifos-oxon in CLRS compared with susceptible strain implied that CLRS had altered AChE target site. These findings are expected to help researchers detect resistance mechanisms in diamondback moth and plan effective control strategies for its management. Further studies seem to be necessary to determine resistance genes involved in chlorpyrifos resistance in *P. xylostella.*

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مکانیسم‌های بیوشیمیایی و الگوهای مقاومت تقاطعی مقاومت به کلرپیریفوس در یک سویه مقاوم شده شب پره پشت الماسی *Plutella xylostella* (Lepidoptera: Plutellidae)

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چکیده

شب پره پشت الماسی یا بید کلم، *Plutella xylostella*، یکی از آفات مهم چلیپاییان در سراسر دنیا و ایران می‌باشد و معمولا با استفاده از حشره‌کش کلرپیریفوس کنترل می‌شود. این آفت به دلیل داشتن طیف وسیعی از ویژگی‌های بیوشیمیایی و رفتاری می‌تواند به سرعت در برابر بسیاری از حشره‌کش‌های شیمیایی مختلف مقاوم شود. به منظور دستیابی به یک طرح مدیریت مقاومتی بهتر، یک سویه مقاوم از شب پره پشت الماسی به حشره‌کش کلرپیریفوس تحت فشار گزینشی در آزمایشگاه ایجاد شد و مقاومت تقاطعی آن به پنج حشره‌کش دیگر و همچنین خصوصیات مقاومتی آن مورد بررسی قرار گرفت. پس از گذشت ۱۵ نسل از فشار گزینشی، جمعیت مقاوم (CLRS) حدود ۳۹/۶۱ برابر نسبت به جمعیت حساس (AL) به کلرپیریفوس مقاوم شد. نسبت مقاومت تقاطعی در جمعیت مقاوم (CLRS) نسبت به جمعیت حساس (AL) در برابر حشره‌کش‌های هگزافلومورون، ایندوکساکارب، تیودیکارب و فلوبندیامید به ترتیب ۱۹/۶۲، ۱۷/۸۴، ۳/۴۳ و ۳/۳۳-برابر بود. همچنین در بررسی مقاومت تقاطعی، هیچ نوع مقاومت تقاطعی در برابر حشره‌کش آبامکتین مشاهده نگردید. اگرچه آزمایش‌های بیوشیمیایی و سینرژیسمی نشان دادند که استرازها در جمعیت مقاوم نقش مهمی را

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در مقاومت به کلرپیریفوس دارند، تفاوتی در فعالیت آنزیم‌های گلوتاتیون اس-ترانسفراز و مونواکسیژناز در جمعیت مقاوم و جمعیت حساس مشاهده نشد. برای تعیین غیر حساس شدن آنزیم استیل کولین استراز، پارامترهای سینتیکی (K_m , V_{max})، و اثر بازدارندگی کلرپیریفوس_اکسان روی این آنزیم مورد بررسی قرار گرفت. میل ترکیبی وکارایی تجزیه‌ای این آنزیم در سویه مقاوم بیشتر از سویه حساس بود. همچنین مشخص شد در سویه مقاوم این آنزیم حساسیت کمتری نسبت به مهارکننده کلرپایریفوس_اکسان دارد. نتایج حاصل از این تحقیق می‌تواند برای استفاده‌کنندگان از سموم شیمیایی موثر باشد و همچنین می‌تواند موجب به تاخیر افتادن توسعه مقاومت در شب پره پشت الماسی شود.

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