

Research Article

Resistance mechanisms of a field population of diamond back moth, *Plutella xylostella* (Lepidoptera: Plutellidae) to current organophosphate pesticides

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Abstract: The toxicological and biochemical properties of four organophosphate (OP) insecticides, chlorpyrifos, diazinon, phosalone and dichlorvos, were examined in terms of the diamond back moth, *Plutella xylostella* (L.) susceptible (Gu-S) and resistant (Kar-R) to OPs. The Kar-R population had significantly high resistance to chlorpyrifos (69.3 fold), medium resistance to diazinon (14.49-fold) and phosalone (10.3-fold), and had less resistance to dichlorvos (5.17-fold) compared to Gu-S population. DEM and TPP reduced Chlopyrifos resistance of Kar-R population as an inhibitor of glutathione S-transferase (GST) and esterases (EST), respectively. Biochemical studies clarified that GST and EST kinetic parameters in the Kar-R population were significantly higher than parameters of Gu-S population. Moreover, this study examined the Kinetics of hydrolysis of acetylthiocholine iodide, butyrylthiocholine iodide as artificial substrates by AChE of resistant and susceptible population. IC₅₀ of monocrotophos, neostigmine bromide and eserine were also determined on AChE of resistant and susceptible populations. Kinetic analysis and inhibition tests indicated that an alteration in AChE of Kar-R population has an effect on both kinetic and inhibition results. The results distinctly showed that multiple mechanisms such as GST, esterases and altered AChE created chlorpyrifos resistance in the Kar-R and insensitivity of AChE is a significant factor for resistance to conventional OP compounds.

Keywords: *Plutella xylostella*, organophosphate pesticides, biochemical mechanisms, detoxification enzymes, acetylcholinesterase

Introduction

Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is one of the most devastating insect pests of cruciferous vegetables and has caused US \$ 27 billion worth of global crop losses and control expenses (Zalucki *et al.*, 2012). Short production time, high fecundity,

seasonal migration patterns and a wide host plant range have turned DBM into a significant agricultural pest. DBM has increased a resistance to conventional insecticides that have been used intensively and extensively for many decades (Sayyed *et al.*, 2008). Available insecticides have been rendered ineffective against the DBM, an urgent action is required to manage the resistance and expand the lifespan of current insecticides.

Organophosphates (OPs) are mostly used to control the agricultural pests in Iran. The OP insecticides cause toxicity by unalterable inhibition of acetylcholinesterase (AChE), which

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reduces the excitatory neurotransmitter, acetylcholine. AChE is the main target site of both organophosphate and carbamate insecticides, which blocks the enzyme activity by covalently phosphorylating and carbamylating the serine remains contain the active site gap (Corbett, 1974; Fournier, 1993). Chlorpyrifos is a wide spectrum, non-systemic and relatively toxic organophosphate insecticide that has been commonly used for more than 40 years to control many different insect pests. In California, on the average 1.45 million pounds of active ingredient of chlorpyrifos products was used annually between 2002 and 2012 (Lori and Peter, 2014). Many crop insect pests, such as *Bemisia tabaci* (Zhang *et al.*, 2012), *Laodelphax striatellus* (Zhang *et al.*, 2013) and *Sogatella furcifera* (He *et al.*, 2015) had high level resistance to chlorpyrifos. Phosalone, diazinon and dichlorvos are the other OP pesticides which are used widely against *P. xylostella* in Iran. There are some reported cases of high horn fly resistance to diazinon (Beak *et al.*, 2005) and phosalone in *Agonoscyta pistaciae* population (Alizadeh *et al.*, 2014). According to the resistance mechanisms, improved activities of carboxyl esterase (Sun *et al.*, 1978; Noppun *et al.*, 1987) and glutathione S-transferase (Cheng *et al.*, 1983) have been listed as degradation factors, even a decrease in sensitivity of the target enzyme, AChE, to OP has as well been presented (Sudderuddin and Kok, 1978; Noppun *et al.*, 1983). Insensitive AChE has been reported in more than 30 insecticide resistant pest species. The two spotted spider mites were the first reports of OP resistant strains (Smissaert, 1964; Voss and Matsumura, 1964; Kozaki *et al.*, 2001).

This study was undertaken to compare some toxicological and biochemical properties of detoxification enzymes (ESTs and GST) and cholinesterase between two resistant (Kar-R) and susceptible (Gu-S) field populations of *P. xylostella*

Materials and Methods

Insects

The chlorpyrifos-susceptible (GU-S) population was collected from Rasht, Guilan

province, Iran and the chlorpyrifos-resistant (Kar-R) population from Karaj, Alborz province, Iran. The larvae were reared on *Brassica rapae* and the adults were fed on 10% honey-water. Insect populations were maintained at 25 ± 2 °C and 70 ± 10 % relative humidity under a photoperiod of 16:8 h (L: D).

Insecticide and chemicals

Chlorpyrifos (40% EC), diazinon (60% EC), phosalone (35% EC) and dichlorvos (50% EC) were obtained from Bayer and used in the bioassays. Fast blue RR salt was obtained from Fluka (Fluka, Buchs, Switzerland). Aldicarb 98% purity, eserine, neostigmine, carbofuran 99.5% purity, paraoxan and carbaryl, α -naphthyl acetate (α NA), β -naphthyl acetate (β -NA) and α -naphthylbutyrate (α -NB) were purchased from Sigma (www.sigmaaldrich.com). Monocrotophos and phosphamidon with 99.9% purity were obtained from Accustandard (www.accustandard.com). Acetylthiocholine iodide (ATC), S-butylthiocholine iodide (BTC) and 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Wako (www.wako-chem.co.jp). Piperonyl butoxide (PBO), diethyl maleate (DEM), triphenyl phosphate (TPP) and all other chemicals were purchased from Merck (Darmstadt, Germany) and were reagent grade.

Insecticide

Toxicity of insecticides was measured using a standard leaf-dip bioassay with the leaves of cauliflower. Using commercial formulations, further serial dilutions were prepared in distilled water with Triton X-100 (0.1%) as a sticker to obtain five concentrations for each insecticide. The leaves were thoroughly washed in distilled water containing 0.1% Triton X-100 and dried at room temperature. Bioassays were conducted on third-instar larvae of *P. xylostella*. Leaf discs (5 cm) were dipped in separate concentrations (2000-3000-4000-6000, 9000 (Kar-R) and 25-35-60-100, 150 (Gu-S) of chlorpyrifos for 30s and after that treated leaf discs were air dried at room temperature for 1 h. The discs individually were transferred to plastic petri dishes (7 cm diameter). In control, the leaf discs were immersed in distilled

water containing 0.1% Triton X-100. Concentrations covering 10% to 90% mortality and each concentration had eight replicates and ten third instar larvae of the same age were used per replicate. If larvae were not able to move in a coordinated way when touched with a brush, the larvae were considered as dead. Mortality was documented after 48 h and the LC_{50} s and their confidence limits were estimated using POLO-PC software (LeOra Software, 2007).

Synergism assay

To determine synergistic effects, stock solutions (10 g litre^{-1}) of PBO, a MFO inhibitor, DEM, a GST inhibitor, and TPP, an esterase inhibitor, were prepared in 100% acetone (MERK). These solutions were then diluted in distilled water containing $200 \mu\text{l litre}^{-1}$ spreading agent before synergism tests. Synergism was measured using the above described leaf-dip bioassay. Preliminary tests (maximum concentrations that caused no mortality) indicated that the concentrations of PBO, DEM and TPP for the susceptible population, respectively, were 50, 100 and 100 mg litre^{-1} and 100, 180 and 200 mg litre^{-1} for resistant population, respectively. To test the effect of PBO, DEM and TPP on the efficacy of chlorpyrifos, synergist was added to each of various concentrations of chlorpyrifos. Control for each strain were used. Mortality was scored after 48 h. LC_{50} values were calculated by Probit regression using POLO-PC software (Kodwo and Tanaka, 2005). The synergism ratio (SR), which is a measure of synergistic effect, was calculated by dividing the LC_{50} of the population treated with chlorpyrifos alone by the LC_{50} of population treated with chlorpyrifos plus synergist.

Enzyme assays

EST

EST assays were performed according to the method of Van Asperen (1962). α -NA and β -NA were used as substrates. For esterase enzyme assay, ten third instar larvae were homogenized in $600 \mu\text{l}$ of 0.02 M phosphate buffer, containing 0.05% (v/v) Triton X-100 and pH 7.4 on ice, then were centrifuged at $11,000 \times g$ for 10 minutes at 4°C . The kinetic parameters of the EST enzyme

were analyzed using different concentrations (0.2-0.4- 0.8-1.6-3.2- 6.4 mM) of the substrates. $12.5 \mu\text{l}$ of supernatant was mixed with $50 \mu\text{l}$ of substrates, $112.5 \mu\text{l}$ of the phosphate buffer and after all $50 \mu\text{l}$ of 0.2% Fast Blue RR salt was added to mixture. The naphthol production was monitored using a microplate reader (Awareness Technology Inc, Stat Fax 3200®) as a kinetic mode by assessing absorbance at 450 and 540 nm for α -NA and β -NA substrates, respectively. To enable calculation of the amount of naphthol produced in the process of esterase assay, a standard curve of absorbance for determining the amount of naphthol produced was created. The kinetic parameters were determined from Lineweaver-Burk plots. All measurements were done in triplicate.

GST

Ten third-instar larvae were homogenized in $400 \mu\text{l}$ of 0.02 M phosphate buffer, pH 7.4. Enzyme preparation was same as that pointed out above for esterase but without Triton X100. Subsequently, the homogenates were centrifuged for 10 minutes in $11,000 \times g$ at 4°C . Activity was measured using 1-chloro-2,4-dinitrobenzene (with CDNB) and reduced glutathione (GSH) as substrates according to Habig *et al.* (1974) with slight modifications. For the test, $15 \mu\text{l}$ enzyme, $100 \mu\text{l}$ CDNB (1.2 mM) and $100 \mu\text{l}$ GSH (10 mM) were added to microplate. Enzyme activity was measured by monitoring the fluctuation in absorbance at 340 nm for 10 minutes with a microplate reader (Awareness Technology Inc, Stat Fax® 3200). Different concentrations of CDNB (0.01-5 mM) at fixed concentration of GSH (10 mM) were used to determine kinetic parameters, the K_m and V_{max} values were estimated using Lineweaver-Burk plots.

AChE assay

AChE activity and its kinetic parameters were measured with two artificial substrates, ATC and BTC, along with the modified method of Ellman *et al.* (1961). Increase in absorbance was recorded at 415 nm using a microplate, as described by Moores *et al.* (1988). One

thousand third instar larvae of DBM were collected and homogenized, on ice, in 30 ml (15 larvae in 500 μ l) of 0.1 M phosphate buffer (pH 7) containing 0.5% Triton X-100 with a glass tissue grinder. The homogenate was centrifuged at $11,000 \times g$ and 4 °C for 10 min and then supernatant used as enzyme solution. The reaction mixture contained 100 μ l phosphate buffer (20 mM, pH 7.4), 20 μ l DTNB (5, 5-dithio-bis 2-nitrobenzoic acid), 40 μ l ATC or BTC and 40 μ l of enzyme solution. The AChE activity was determined (every 5 min for 35 min) by measuring OD at 415 nm, using a microplate reader (Awareness technology Stat fax 3200®) Kinetic parameters (K_m and V_{max}) of AChE and activities were measured by various concentrations (0.5-50 mM) of ATC and BTC, and a double reciprocal plot was generated.

Total protein assay

Protein concentration was determined by the Bradford assay (Bradford, 1976) using BSA (Bovine serum albumin) as a standard protein. To determine the total protein, 10 μ l of enzyme extract was added to 500 μ l of Bradford reagent and the absorbance was read at 630 nm.

Inhibition assay of AChE

For inhibition assay, the enzyme solution was pre incubated with various concentrations (0.5-50 mM) of monocrotophos, eserine, neostigmine bromide for 10 min at room temperature. Afterward buffer and DTNB were added to mixture. Residual activity was assessed by measuring absorbance at 412 nm kinetically using the standard assay method as mentioned above. I_{50} value was assessed by probit analysis (Finney, 1971) and Sigma plot software. Experiments were done in triplicate. The K_m and V_{max} values were estimated with Lineweaver-Burk plots.

Statistical analysis

The mean values of the recorded data was subjected to analysis of variance (ANOVA), followed by Tukey's test when significant differences were found at $P = 0.05$ using SAS program (SAS Institute, 2004).

Results

Resistance level to OP insecticides

When compared to the susceptible population, the Kar-R population exhibited 69.3-, 14.49-10 and 5- fold resistance ratio to chlorpyrifos, diazinon, phosalone and dichlorvos, respectively. Among the four organophosphorus pesticides tested, the Kar-R population had the highest resistance to chlorpyrifos (Table 1).

Synergistic effect

The effects of three inhibitors on chlorpyrifos toxicity in the Kar-R and susceptible populations are shown in Table 2. In Kar-R population, the synergistic ratios of DEM, PBO, TPP on chlorpyrifos toxicity were 1.26, 1- and 1.56-fold, respectively. In susceptible moth, synergistic ratios of DEM, PBO and TPP on chlorpyrifos toxicity were observed as 1.3, 1.12 and 1.06 -fold, respectively. However, PBO have the similar synergism in the two populations (1.06 and 1-fold). Result shows TPP has higher synergism in the Kar-R population (1.56-fold), which implies that ESTs could be involved somewhat in the metabolic detoxification of chlorpyrifos. The resistance was not completely suppressed by synergists. Therefore, it is possible to consider another mechanism of resistance besides the metabolism by GST.

Detoxification enzymes

Results of kinetic parameters of EST, GST and MFO in resistant and susceptible populations of *P. xylostella* are summarized in Table 3. Both α -NA and β -NA were used to measure esterase activities. The specificity constant (V_{max}/K_m) and specific activity (SA) of esterase for both model substrates was more noticeable in the resistant population than that in Gu-S population. V_{max}/K_m values for male and female of the Kar-R population was higher than that of Gu-S population when α -NA and β -NA used as substrate. The GST activity was measured using CDNB as a substrate (Table 3). GST activity in Kar-R population was higher than that of Gu-S population and their specificity constants were for females 1.9-fold and for males 1.26 fold that in the Gu-S population, respectively.

AChE activity and its kinetic parameters

Kinetic parameters of AChE and its activity are shown in Table 4. As shown, the *Km* values of Gu-S Male and female populations were higher than that of Kar-R population for

the two (ATC, BTC) substrates. It appears that increase in *Km* in Gu-S population led to low affinity of the enzyme for the substrate without changing the reaction characteristics (Fig. 1).

Table 1 Response of susceptible (Gu-S) and Resistant (Kar-R) populations of *Plutella xylostella* to chlorpyrifos, diazinon, phosalone and dichlorvos.

| Population | Insecticide | LC ₅₀ (µg/ml) | 95% CI ¹ | Slope (SE) | χ ² (df) | RR ² |
|------------|--------------|--------------------------|---------------------|-------------|---------------------|---------------------|
| Kar-R | Chlorpyrifos | 4300.42 | 2722.6 - 7276.3 | 2.75 (0.42) | 4.21 (3) | 69.30 (53.2 - 90.4) |
| Kar-R | Diazinon | 2899.80 | 2385.7 - 3617.9 | 2.63 (0.43) | 1.95 (3) | 14.49 (8.6 - 20.4) |
| Kar-R | Phosalone | 987.79 | 673.6 - 1433.6 | 2.57 (0.51) | 5.11 (3) | 10.30 (7.5 - 14.1) |
| Kar-R | Dichlorvos | 658.44 | 436.1 - 914.9 | 1.75 (0.29) | 0.66 (3) | 5.17 (3.5 - 7.7) |
| Gu-S | Chlorpyrifos | 61.97 | 51.1 - 95.7 | 2.75 (0.42) | 1.13 (3) | 1 |
| Gu-S | Diazinon | 200.00 | 132.2 - 183.9 | 3.84 (0.59) | 1.14 (3) | 1 |
| Gu-S | Phosalone | 92.28 | 72.4 - 112.1 | 3.00 (0.57) | 0.29 (3) | 1 |
| Gu-S | Dichlorvos | 122.68 | 103.3 - 149.2 | 3.00 (0.48) | 1.05 (3) | 1 |

¹ 95% confidence interval.

² RR: Resistance ratio = LC₅₀ of the resistant population/LC₅₀ of the susceptible population.

Table 2 Effect of piperonyl butoxide (PBO), diethyl maleate (DEM) and triphenyl phosphate (TPP) on chlorpyrifos toxicity in *Plutella xylostella* populations.

| Population | Synergis | N | LC ₅₀ (%95 CI) (mg/ml) | Slope (SE) | χ ² (df) | SR (%95 CI) |
|------------|----------|-----|-----------------------------------|-------------|---------------------|--------------------|
| Gu-S | without | 400 | 61.97 (51.10 - 95.65) | 2.75 (0.42) | 1.13 (3) | --- |
| | PBO | 400 | 58.21 (47.34 - 91.89) | 2.80 (0.44) | 1.10 (3) | 1.06 (0.86 - 1.40) |
| | DEM | 400 | 55.00 (45.50 - 89.44) | 2.80 (0.44) | 0.61 (3) | 1.12 (0.88 - 1.50) |
| | TPP | 400 | 47.18 (35.49 - 56.30) | 3.80 (0.80) | 2.20 (3) | 1.30 (0.97 - 1.69) |
| Kar-R | without | 400 | 4300.42 (3722.6 - 7276.3) | 2.75 (0.42) | 4.21 (3) | --- |
| | PBO | 400 | 4258.46 (3279.0 - 5280.2) | 3.10 (0.51) | 2.40 (3) | 1.00 (0.78 - 1.34) |
| | DEM | 400 | 3391.00 (2617.0 - 4094.0) | 3.30 (0.62) | 0.30 (3) | 1.26 (1.04 - 1.66) |
| | TPP | 400 | 2742.80 (1869.2 - 3463.9) | 3.20 (0.76) | 1.71 (3) | 1.56 (1.21 - 2.50) |

Synergistic Ratio (SR) = LC₅₀ of insecticide/LC₅₀ of (synergist + insecticide). Gu-S and Kar-R indicate susceptible and resistant populations respectively.

Table 3 Kinetic parameters and specific activity of detoxification enzymes in susceptible (Gu-S) and resistant (Kar-R) populations of *Plutella xylostella*.

| Sex | Population | Enzyme | Substrate | Special activity ± SE (nmol/min.mg protein) | | | |
|--------|------------|--------|-----------|---|---------------|----------------|----------------|
| | | | | fSA | Km | Vmax | Vmax/Km |
| Female | Kar-R | EST | α-NA | 0.44 ± 0.02a | 0.78 ± 0.09a | 0.13 ± 0.005a | 0.16 ± 0.027a |
| | | | β-NA | 0.17 ± 0.003a | 0.65 ± 0.007a | 0.09 ± 0.006a | 0.13 ± 0.001a |
| | | GST | CDNB | 2.10 ± 0.009 a | 2.20 ± 0.008a | 46.00 ± 6.000a | 21.00 ± 2.000a |
| | Gu-S | EST | α-NA | 0.23 ± 0.009b | 1.30 ± 0.01b | 0.10 ± 0.007b | 0.07 ± 0.008b |
| | | | β-NA | 0.12 ± 0.005b | 0.85 ± 0.01b | 0.09 ± 0.005b | 0.10 ± 0.008b |
| | | GST | CDNB | 1.28 ± 0.005b | 1.70 ± 0.008b | 18.40 ± 1.000b | 11.00 ± 1.000b |
| Male | Kar-R | EST | α-NA | 0.42 ± 0.017a | 0.60 ± 0.04a | 0.10 ± 0.002a | 0.16 ± 0.030a |
| | | | β-NA | 0.16 ± 0.005a | 0.50 ± 0.009a | 0.07 ± 0.002a | 0.14 ± 0.003a |
| | | GST | CDNB | 2.40 ± 0.02a | 2.20 ± 0.006a | 40.00 ± 3.000a | 19.00 ± 1.000a |
| | Gu-S | EST | α-NA | 0.20 ± 0.005b | 1.10 ± 0.008b | 0.08 ± 0.006b | 0.07 ± 0.007b |
| | | | β-NA | 0.14 ± 0.008b | 0.80 ± 0.009b | 0.09 ± 0.005b | 0.11 ± 0.009b |
| | | GST | CDNB | 1.30 ± 0.009b | 1.66 ± 0.006b | 25.00 ± 3.000b | 15.00 ± 2.500b |

Means of kinetic parameters followed by the same letters in each column are not significantly different (Tukey's test, P < 0.05).

EST: Esterse, GST: glutation-S-transferase, α-NA: α- naphthyl acetate, β-NA: β-naphthyl acetate, CDNB: 1-chloro-2, 4 dinitrobenzene special activity (nmol/min.mg protein) ± SE.

Table 4 Substrate specificities and kinetic parameters (mean \pm SE) of AChE from susceptible (Gu-S) and resistant (Kar-R) populations of *Plutella xylostella*.

| Sex | Population | Substrate | Special activity \pm SE (nmol/min.mg protein) | | | |
|--------|------------|-----------|---|---------------------|--------------------|----------------------|
| | | | SA | Km | Vmax | Vmax/Km |
| Female | Gu-S | ATC | 0.570 \pm 0.080a | 0.650 \pm 0.040a | 0.058 \pm 0.010a | 0.089 \pm 0.018a |
| | | BTC | 0.029 \pm 0.006a | 16.130 \pm 0.050a | 0.021 \pm 0.006a | 0.0013 \pm 0.0008a |
| | Kar-R | ATC | 0.310 \pm 0.050b | 0.222 \pm 0.010b | 0.028 \pm 0.009b | 0.126 \pm 0.009b |
| | | BTC | 0.015 \pm 0.008b | 8.280 \pm 0.080b | 0.027 \pm 0.004b | 0.0032 \pm 0.0001b |
| Male | Gu-S | ATC | 0.520 \pm 0.100a | 0.570 \pm 0.020a | 0.041 \pm 0.005a | 0.071 \pm 0.085a |
| | | BTC | 0.022 \pm 0.004a | 16.100 \pm 0.050a | 0.018 \pm 0.003a | 0.0011 \pm 0.0005a |
| | Kar-R | ATC | 0.280 \pm 0.010b | 0.190 \pm 0.008b | 0.020 \pm 0.008b | 0.100 \pm 0.005b |
| | | BTC | 0.013 \pm 0.004b | 9.140 \pm 0.090b | 0.027 \pm 0.005b | 0.0029 \pm 0.0003b |

Means of kinetic parameters followed by the same letters in each column are not significantly different (Tukey's test, $P < 0.05$).

ATC: Acetylthiocholine iodide, BTC: Butyrylthiocholine iodide specific activity.

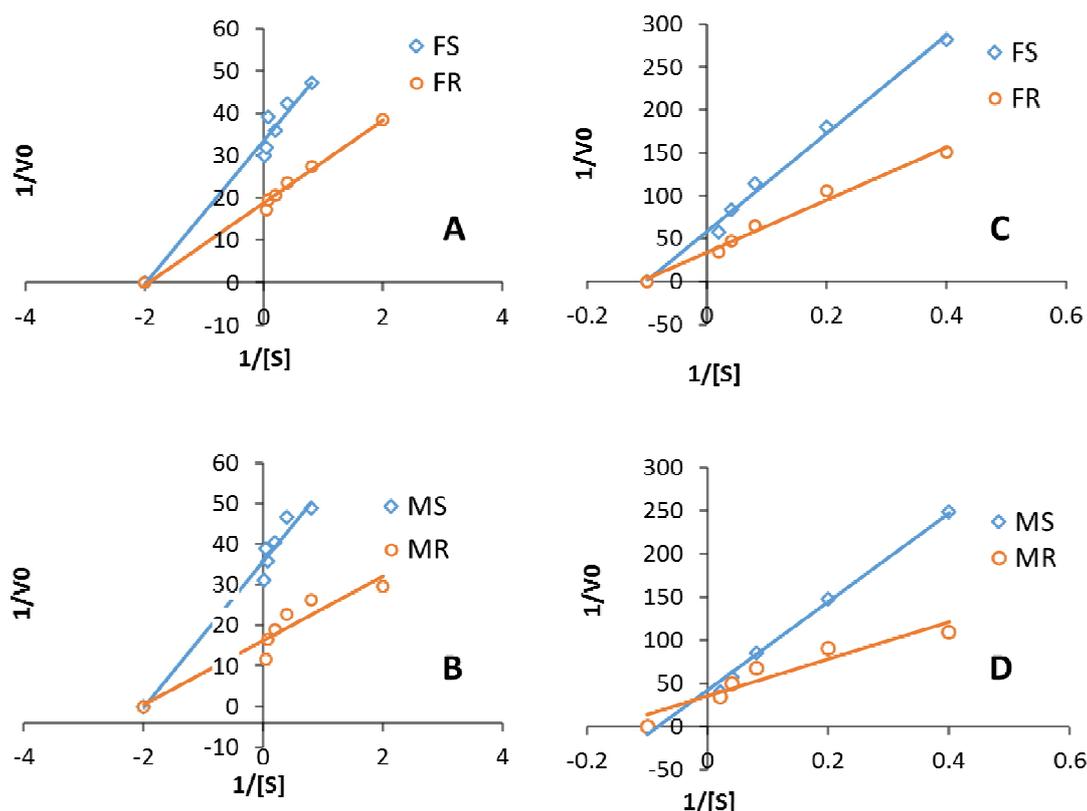


Figure 1 Lineweaver-Burk plots of ATC and BTC hydrolysis by AChE in susceptible (Gu-S) and resistant (Kar-R) populations of *Plutella xylostella*. Hydrolysis of ATC by female of Gu-S (FS) and Kar-R populations (FR) (A), and male of Gu-S (MS) and Kar-R populations (MR) (B). Hydrolysis of BTC by Female of Gu-S and Kar-R populations (C), and Male of Gu-S and Kar-R populations (D).

Inhibition of AChE

The effects of OPs and carbamates on AChE activity from mass homogenates of resistant and susceptible populations were determined, and the results are shown in Table 5 and Fig. 2. Compared with I_{50} values for the Gu-S, the resistant population (Kar-R) demonstrated 11, 11, 5, 3 and 2 -fold resistance in female and 10.2, 10.2, 4.2, 3 and 2- fold in male to monocrotophos, neostigmine and eserine, carbaryl and phosphamidon, separately. In contrast, there is no noteworthy difference in inhibition of AChE by paraoxan, carbofuran and aldicarb among Gu-S and Kar-R populations. These results imply that resistance to chlorpyrifos in Kar-R population causes simultaneous insensitivity to other inhibitors such as monocrotophos, neostigmine and eserine, carbaryl and phosphamidon.

Discussion

DBM has gained resistance against many insecticides because of its short lifespan, high

reproduction, and the high selection pressure with insecticides (Zhao *et al.*, 2002, Sayyed and Wright, 2006, Pu *et al.*, 2010, Zhou *et al.*, 2011), biotic potential, overlapping generations and the high migratory ability (Talekar and Shelton, 1993, Sarfraz *et al.*, 2006). In this study, a field population (Kar-R) of the DBM collected from Karaj County, Alborz, Iran, was proved to have marginal to high resistance to selected OP insecticides compared to the susceptible population (Gu-S). The findings expressed a high degree of resistance to chlorpyrifos compared to diazinon, phosalone and dichlorvos (Table 1).

For chlorpyrifos, LC_{50} value of Kar-R was about 69-fold higher than the susceptible population. Chlorpyrifos has been used to control DBM and other insect pests for many years in Iran and the world (Pasteur and Sinègre, 1978; Milio *et al.*, 1987; Rust and Reiersen, 1991; Archer, 1994; Guides *et al.*, 1996; Liu *et al.*, 2005; Curtis and Pasteur, 2009; Zhang *et al.*, 2012).

Table 5 I_{50} values of *in vitro* inhibition of AChE activity in the chlorpyrifos -resistant and susceptible populations of *Plutella xylostella*.

| Sex | Inhibitor | Guilan | | Karaj | | IR ¹ |
|--------|---------------|----------------------|---|----------------------|---|-----------------|
| | | IC ₅₀ | 95% CL | IC ₅₀ | 95% CL | |
| Male | Aldicarb | 6×10^{-5} | $3 \times 10^{-5} - 1.2 \times 10^{-4}$ | 5.5×10^{-5} | $2.8 \times 10^{-5} - 10^{-4}$ | 1.0 |
| | Monocrotophos | 6×10^{-5} | $3 \times 10^{-5} - 1.2 \times 10^{-4}$ | 7×10^{-4} | $2.5 \times 10^{-4} - 1.2 \times 10^{-4}$ | 11.0* |
| | Eserine | 7×10^{-8} | $10^{-8} - 10^{-7}$ | 4×10^{-7} | $10^{-7} - 8 \times 10^{-7}$ | 5.0* |
| | Neostigmine | 7×10^{-7} | $3 \times 10^{-7} - 1.4 \times 10^{-6}$ | 8×10^{-6} | $2 \times 10^{-6} - 3 \times 10^{-5}$ | 11.0* |
| | Phosphamidon | 6.1×10^{-5} | $4 \times 10^{-5} - 1.17 \times 10^{-5}$ | 1.2×10^{-4} | $10^{-4} - 5.4 \times 10^{-3}$ | 2.0 |
| | Carbofuran | 5×10^{-7} | $4 \times 10^{-7} - 8 \times 10^{-7}$ | 5×10^{-7} | $4 \times 10^{-7} - 7.7 \times 10^{-7}$ | 1.0 |
| | Paraoxan | 10^{-5} | $4 \times 10^{-6} - 3.2 \times 10^{-4}$ | 3×10^{-5} | $10^{-5} - 8 \times 10^{-5}$ | 1.0 |
| | Carbaryl | 4×10^{-6} | $2.6 \times 10^{-6} - 6.2 \times 10^{-6}$ | 9×10^{-6} | $10^{-6} - 1.6 \times 10^{-5}$ | 3.0 |
| Female | Aldicarb | 6×10^{-5} | $3 \times 10^{-5} - 1.2 \times 10^{-4}$ | 5.3×10^{-5} | $2.9 \times 10^{-5} - 10^{-4}$ | 1.0 |
| | Monocrotophos | 6.1×10^{-5} | $3 \times 10^{-5} - 1.2 \times 10^{-4}$ | 4.6×10^{-4} | $1.8 \times 10^{-4} - 3 \times 10^{-5}$ | 10.2* |
| | Eserine | 7×10^{-8} | $10^{-8} - 10^{-7}$ | 3×10^{-7} | $6.8 \times 10^{-8} - 5 \times 10^{-7}$ | 4.2* |
| | Neostigmine | 6.8×10^{-7} | $3 \times 10^{-7} - 1.4 \times 10^{-6}$ | 7×10^{-6} | $2 \times 10^{-6} - 3 \times 10^{-5}$ | 10.2* |
| | Phosphamidon | 6×10^{-5} | $4 \times 10^{-5} - 1.17 \times 10^{-5}$ | 3×10^{-5} | $10^{-5} - 6 \times 10^{-5}$ | 2.0 |
| | Carbofuran | 5×10^{-7} | $4 \times 10^{-7} - 8 \times 10^{-7}$ | 5×10^{-7} | $4 \times 10^{-7} - 7.7 \times 10^{-7}$ | 1.0 |
| | Paraoxan | 10^{-5} | $4 \times 10^{-6} - 3.2 \times 10^{-4}$ | 3×10^{-5} | $10^{-5} - 8 \times 10^{-5}$ | 1.0 |
| | Carbaryl | 4×10^{-6} | $2.6 \times 10^{-6} - 6.2 \times 10^{-6}$ | 9×10^{-6} | $10^{-6} - 1.6 \times 10^{-5}$ | 3.0 |

¹ Insensitivity Ratios (IR) = IC_{50} of Kar-R population/ IC_{50} of Gu-S population.

* Statistically significant at 95% level ($P < 0.05$).

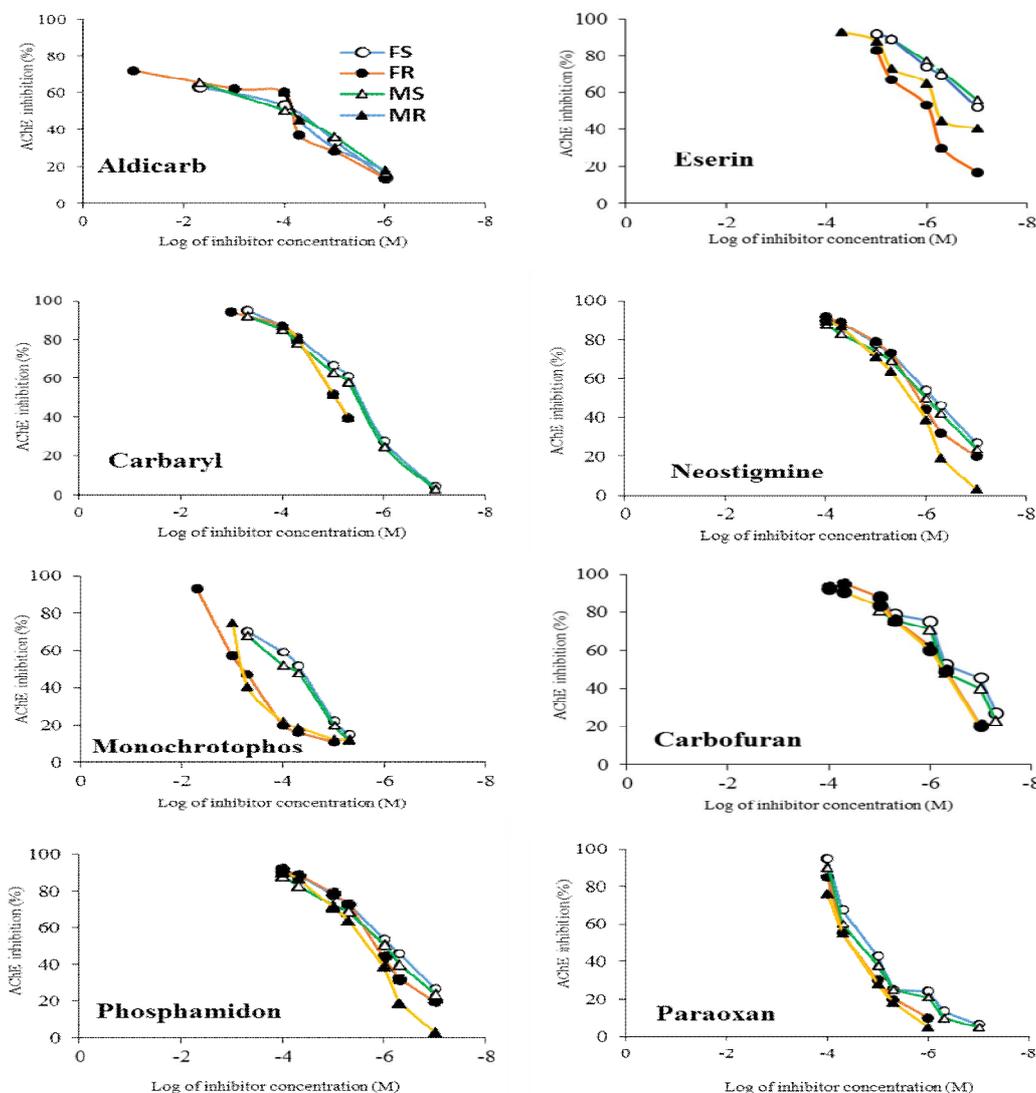


Figure 2 Inhibition of acetylcholinesterase (AChE) in female of susceptible, Gu-S, (FS) and resistant, Kar-R, (FR), and male of Gu-S (MS) and Kar-R (MR) populations of *Plutella xylostella* by some OPs and carbamate pesticides.

Some researchers have reported the resistance of this pest to some OP compounds. Sun *et al.* (1978) reported 14-fold resistance to diazinon in Taiwan and Yu and Neguyen (1992) published 73- and 21-fold resistance of DBM respectively to diazinon and chlorpyrifos in Florida. Enhanced activities of GST (Cheng *et al.*, 1983) and carboxylesterase (Sun *et al.*, 1978; Noppun *et al.*, 1987) have been explained as degradation factors related to the OP-resistance and even modification

of acetylcholinesterase (AChE) to an insensitive form of the target enzyme (Sudderuddin and Kok, 1978; Noppun *et al.*, 1983). Synergists with blocking definite insecticide metabolic enzymes are used to improve the toxicities of insecticides against insect pests and the degree of synergism can suggest whether or not conceived detoxification enzymes metabolize the pesticides (Yang *et al.*, 2001). In this research, we used three synergists (TPP, an EST inhibitor; PBO, a P450

inhibitor; and DEM, a GST inhibitor). As shown in Table 2, synergism effect triggered a reduction in LC_{50} values of OP on resistant population (SR: 1.26 and 1.56-fold for DEM and TPP, respectively). Thus, these results indicated that metabolic enzymes especially EST play a role in chlorpyrifos resistance. We compared the EST and GST kinetic parameters and specific activities between the Kar-R and Gu-S populations to confirm the possible contributions of these major detoxification enzymes in chlorpyrifos resistance in the Kar-R. Our results expressed that EST and GST activities in the Kar-R were significantly higher than activities in the Gu-S. In fact, results expressed that EST and GST cannot be the only cause that involves in chlorpyrifos resistance, therefore other mechanisms such as target site resistance should be involved in it. Kinetic parameters of this enzyme and inhibitory effect of monocrotophos, neostigmine and eserine as AChE inhibitor were studied to determine the role of AChE insensitivity in resistance mechanisms. There was an insensitive AChE to OP insecticides in several insects, including mosquitos, housefly (Kozaki *et al.*, 2009; Walsh *et al.*, 2001) aphids (Gao *et al.*, 2002; Andrews *et al.*, 2014), *Drosophila* (Mutero *et al.*, 1994; Menozzi *et al.*, 2004), and spider mites (Zamani *et al.*, 2014) and even has been observed in *P. xylostella* on diazinon (Sun *et al.*, 1978), phenthoate (Noppun *et al.*, 1987), chlorpyrifos (Cho *et al.*, 2006), prothiofos (Beak *et al.*, 2005; Lee *et al.*, 2007). Our study indicated that the specific activity of AChE related to ATC was 1.83-fold higher in the Gu-S (female) and 1.85 (male) compared to the Kar-R. KM and V_{max} values of AChE in Kar-R are about two fold less compared to those from the Gu-S population. That shows AChE had different dependency to substrates (ATC, BTC). Different studies show some different results of AChE KM and V_{max} values (Raymond *et al.*, 1986; Zhu and Clark, 1994; Stumpf *et al.*, 2001; Gao and Zhu, 2002; Ren *et al.*, 2002; Yu, 2006; Chai *et al.*, 2007; Hsu *et al.*, 2008; Alizadeh *et al.*, 2014). Female and male in Kar-R population with almost same specific activity, KM and V_{max} values probably both are involved in resistance (Table 4). These results indicated that male and

female of Kar-R population have modified acetylcholinesterase and that AChE in both sexes is assumed to be related to OP resistance. Decreased sensitivity of AChE has also been determined as one of the common mechanisms of resistance to OP compounds in many insects (Huang and Han, 2007). Our kinetic analyses clearly showed that AChE in the Kar-R population was significantly different from the Gu-S AChE. Such differences were indicated by not only the reduced sensitivity levels to different OP inhibitors (Table 5), but also the decreased dependency on ATC and BTC (Table 4). These results highly expressed that reduced sensitivity of AChE also played a significant role to increase OP resistance in the Kar-R. It is concluded that chlorpyrifos resistance in the Kar-R was partly due to the EST and GST mediated metabolic detoxification as shown by significant synergistic effect of DEM and TPP on the toxicity of chlorpyrifos, and significantly increased GST and EST activities. In addition, increased activity and reduced sensitivity of AChE also increased chlorpyrifos resistance in the Kar-R.

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سازوکارهای مقاومت جمعیت شب‌پره پشت‌الماسی *Plutella xylostella* (Lepidoptera: Plutellidae) به حشره‌کش‌های ارگانوفسفره رایج

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چکیده: ویژگی‌های سم‌شناسی و بیوشیمیایی چهار حشره‌کش ارگانوفسفره، کلرپیریفوس، دیازینون، فوزالون و دیکلروس در جمعیت‌های شب‌پره‌پشت‌الماسی یا بید کلم حساس (Gu-S) و مقاوم (Kar-R) به سموم ارگانوفسفره مورد بررسی قرار گرفت. جمعیت Kar-R مقاومت قابل توجهی به کلرپیریفوس (۶۹.۳ برابر)، مقاومت متوسط به دیازینون (۴۹.۴ برابر) و فوزالون (۱۰.۳ برابر) و مقاومت کم‌تر نسبت به دیکلروس (۵.۱۷ برابر) در مقایسه با جمعیت Gu-S داشت. دی‌اتیل‌مالئات و تری‌فنیل‌فسفات به‌عنوان یک بازدارنده به ترتیب برای گلوکوتایون S- ترانسفراز (GST) و استراز (EST) سبب کاهش میزان مقاومت به کلرپیریفوس در جمعیت Kar-R گردید. مطالعات بیوشیمیایی نشان داد که پارامترهای سینتیکی گلوکوتایون اس-ترانسفراز و استراز در جمعیت Kar-R به‌طور معنی‌داری بیش‌تر از پارامترهای جمعیت Gu-S بوده است. علاوه بر این، در این تحقیق سینتیک هیدرولیز استیل تیوکولین یدید و بوتیرل تیوکولین یدید به‌عنوان زیر نهشت‌های مصنوعی استیل کولین استراز در جمعیت‌های مقاوم و حساس مورد بررسی قرار گرفت. IC_{50} مونوکلروتوفوس، نئواستیگمین بروماید و اسرین نیز در استیل کولین استراز جمعیت مقاوم و حساس تعیین شد. تجزیه و تحلیل سینتیکی و تست‌های بازدارندگی نشان داد که تغییرات در استیل کولین استراز جمعیت Kar-R بر نتایج سینتیکی و بازدارندگی مؤثر بوده است. نتایج به‌وضوح نشان داد که سازوکارهای متعددی نظیر گلوکوتایون اس- ترانسفراز، استرازها و استیل کولین استراز تغییر یافته باعث ایجاد مقاومت به کلرپیریفوس در جمعیت Kar-R و عدم حساسیت استیل کولین استراز می‌شود که عامل مهمی برای مقاومت در برابر ترکیبات ارگانوفسفره مرسوم است.

واژگان کلیدی: شب‌پره‌پشت‌الماسی، حشره‌کش‌های ارگانوفسفره، سازوکارهای بیوشیمیایی، آنزیم‌های

سم‌زدا، استیل کولین استراز