

Resistance Mechanisms to Chlorpyrifos in Iranian Populations of the Two-spotted Spider Mite, *Tetranychus urticae* (Acari: Tetranychidae)

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ABSTRACT

The toxicity of chlorpyrifos to three Iranian populations of two-spotted spider mite, collected from Isfahan (ISR), Yazd (Yz) and Guilan (GUS2) Provinces were surveyed using the residual contact vial bioassay. The bioassay results showed that resistance ratios of ISR and Yz populations were 176.90 and 9.78 fold compared to the GUS2 population, respectively. Determination of esterase and glutathione-S-transferase activity and their kinetic parameters showed that ISR population had the highest specific activity and specificity constant among the studied populations. Besides, the content of mixed function oxidases in ISR population was the highest. However, synergistic effects of Piperonyl Butoxide, Diethyl Maleate and Triphenyl Phosphate showed that metabolic enzymes did not play an important role in resistance to chlorpyrifos in ISR and Yz populations and enhanced activity of esterase, glutathione-S-transferase and content of mixed function oxidases in these populations were probably due to resistance to some other acaricides. To determine the role of acetylcholinesterase insensitivity in resistance mechanisms, kinetic parameters and inhibitory effect of chlorpyrifos-oxon on this enzyme were investigated. The K_m value of acetylcholinesterase was determined as 0.036, 0.04, and 0.050 mM using acetylthiocholine iodide for GUS2, Yz, and ISR populations, respectively. In addition, the insensitivity ratios of chlorpyrifos-oxon on acetylcholinesterase activity were estimated at 23.30 and 2.96 for ISR and Yz populations, respectively. These results confirmed amino acid substitutions in active site of this enzyme and also indicated that resistant population possessed qualitatively altered AChE.

Keywords: Acetylcholinesterase, Biochemical mechanism, Detoxification enzymes, Organophosphate acaricides, Synergists.

INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) is a serious pest and causes crop losses by direct feeding and reducing the photosynthetic rate in severe infestations (Gorman *et al.*, 2001). *T. urticae* has rapidly developed resistance to almost all type of acaricides, including organophosphates (OPs) which were first found in rose's greenhouses in the eastern

United States as early as 1948 (Helle, 1962). About 70% of the pesticides in current use are OP compounds (Ojha *et al.*, 2011). Resistance to OPs, the first chemical group used to control *T. urticae*, occurred a short time after their introduction in the USA and Europe, with the main mechanism being reduced sensitivity of the acetylcholinesterase (AChE) (Van Leeuwen *et al.*, 2009). Anazawa *et al.* (2003) first cloned AChE of *T. urticae* and reported mutations putatively associated with OP resistance. So far, AChE

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insensitivity to OPs in *T. urticae* has been reported in strains from the Netherlands, Germany, the USA, New Zealand, Israel, Korea, Turkey, Egypt, and Greece (Zahavi and Tahori, 1970; Helle, 1984; Cranham and Helle, 1985; Tag El-Din, 1990; Stumpf and Nauen, 2001; Tsagkarakou *et al.*, 2002; Ay and Yorulmaz., 2010; Kwon *et al.*, 2010a and 2012). Although insensitivity to AChE is the main resistance mechanism to OPs, other mechanisms may also be involved in some cases (Van Leeuwen *et al.*, 2009). This was first investigated by Voss and Matsumura (1964) who found that enhanced detoxification by carboxyesterases and phosphatases was involved in OP resistance mechanisms. There is no information about chlorpyrifos resistance in this pest in Iran. More information can play an important role in circumventing problems associated with acaricide resistance and assist in rotational choices of chemicals for acaricide mixtures and rotations (Ghadamyari *et al.*, 2008a)

The aim of this study was to investigate the biochemical mechanism involved in chlorpyrifos resistance in *T. urticae*. For this purpose, characteristics of some metabolic enzymes (mixed function oxidase (MFO), esterases (EST) and glutathione-S-transferase (GST) along with the target site insensitivity mechanism mediated by AChE) were compared among the populations. These objectives were addressed by studying on synergistic effects of some inhibitors and determining kinetic parameters of these enzymes and inhibitory effect of chlorpyrifos-oxon on AChE activity.

MATERIALS AND METHODS

Insecticides and Chemicals

Acetylthiocholine iodide (ATC), *S*-butyrylthiocholine iodide (BTC), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), propionylthiocholine iodide (PTC) and chlorpyrifos were purchased from Wako Pure Chemical Industries Ltd. (Japan). Fast blue RR salt was obtained from Fluka

(Fluka, Buchs, Switzerland). Chlorpyrifos-oxon, NADH and NADPH, α -naphthyl propionate (α -NP), α -naphthyl acetate (α -NA), β -naphthyl acetate (β -NA) and α -naphthyl butyrate (α -NB) were purchased from Sigma (Sigma, St. Louis, MO. USA). Also, 3, 3', 5, 5'-tetramethyl benzidine (TMBZ) was purchased from Panreac (Spain). PCR Purification Kit was provided by Bioneer (UK). Piperonyl Butoxide (PBO), Diethyl Maleate (DEM), Triphenyl Phosphate (TPP), Cetyltrimethylammonium bromide (CTAB) and all other chemicals were from Merck (Darmstadt, Germany) and were reagent grade.

Mite Populations

The resistant (ISR) and moderately resistant (Yz) populations were collected from infested rose grown in a greenhouse in Isfahan and Yazd, respectively. These populations had been sprayed with chlorpyrifos for several generations in the greenhouse. A population that was relatively more susceptible to acaricides (GUS2) was collected from *Vigna unguiculata* (L.) in Guilan province without any exposure to acaricides and maintained in laboratory for 5 years without being subjected to any chemical agent. The mites were maintained on bean plants (*Vigna unguiculata*) in plastic boxes under greenhouse condition ($25\pm 2^\circ\text{C}$, $60\pm 10\%$ RH). Our previous results have shown that ISR is a multiple resistant population and have developed resistance to abamectin and fenazaquin (Memarizadeh *et al.*, 2011; Mahdavi Moghaddam and Ghadamyari, 2012). Also, the Yz population has exhibited resistance to fenazaquin (Mahdavi Moghaddam and Ghadamyari, 2012).

DNA Extraction

DNA was extracted from five homogenized fresh female mites by modified CTAB extraction method (Ros and Breeuwer, 2007) for GUS2 and Yz populations and using

protocol described by Li *et al.* (2010) for ISR population. In a typical PCR procedure, the DNA thermal cycler (Applied Biosystems, Foster city, CA USA) was programmed as follows: Initial denaturation step at 94°C for 4 minutes, 35 cycles of denaturation at 93°C for 1 minute, annealing at 49°C for 1 minute, extension at 72°C for 90 seconds and a final extension at 72°C for 5 minutes. The specific primers used for amplification and sequencing of COI genes previously designed by Navajas *et al.* (1996): 5'-TGATTTTTGGTCACCCAGAAG-3' and 5'-TACAGCTCCTATAGATAAAAC-3'. The PCR products were then recovered from the agarose gels (1% (W/V)) and purified using a PCR Purification Kit (Bioneer, UK) according to the procedures recommended by the manufactures.

Sequence Analyses

The purified PCR products were sequenced in both directions by MWG (Eurofins MWG operan, Germany). Similarity searches were performed using BLAST N through the NCBI server (Altschul *et al.*, 1997). The multiple sequence alignments were performed with CLUSTAL W program (Thompson *et al.*, 1994). The nucleotide sequences were submitted to the GenBank.

Nucleotide Sequence Accession Numbers

The sequences are submitted in the GenBank under the following accession numbers: HQ732264, HQ732265 and HQ732266 for Yz, ISR and GUS2, respectively.

Determination of Median Lethal Concentration (LC₅₀)

Using the residual contact vial (RCV) bioassay (Kwon *et al.*, 2010), the toxicity of

chlorpyrifos to three populations of the *T. urticae* was determined. Chlorpyrifos was dissolved in acetone to various concentrations (0.125-100 mg AI l⁻¹). 5-ml glass vials (7×1.1 cm) were coated with 150 µl of chlorpyrifos solution using a rolling under a fume hood for 1 h. Fifteen adult female mites (0-24 hours hold) were transferred into each chlorpyrifos-coated vials and mortality was determined after 10 hours treatment. All the bioassays were conducted in triplicate. The criterion for death was that a mite did not move its appendages when prodded with a camel's hair brush. The LC₅₀ values and 95% confidence limits were calculated using POLO-PC program (LeOra Software, 1987).

Synergistic Effect

To determine synergistic effects, glass vials were coated with 150 µl of each synergist (PBO, a MFO inhibitor, TPP, an esterase inhibitor or DEM, a glutathione-S-transferases inhibitor) in the presence of various concentrations of chlorpyrifos solutions as final concentration of synergist was 200 mg AI/l. The selection of 200 mg AI l⁻¹ concentration for synergists were based on preliminary synergists bioassay results and such synergist concentration was expected to result in less than 10% mortality (Alizadeh *et al.*, 2012). Bioassays were then conducted using RCV method, as described above.

Determination of Enzyme Activity and Kinetic Parameters

EST: General EST assays were performed according to the method of Van Asperen (1962). α-NA, β-NA, α-NB and α-NP were used as substrates. Fifty female adults were homogenized in 500 µl of 0.2 M phosphate buffer, pH 7.0, containing 0.05% (v/v) Triton X-100 on ice. They were centrifuged at 10,000g for 15 minutes at 4°C. The kinetic parameters of the enzyme were



investigated using different concentrations of the substrates. 50 μ l of substrate was added to the microplate containing 12.5 μ l supernatant, 112.5 μ l of the buffer and 50 μ l of Fast Blue RR salt at room temperature. After mixing the naphthol production was monitored by measuring absorbance at 450 and 540 nm for α -NA and β -NA, respectively, and at 630 nm for α -NB and α -NP in a microplate reader (Awareness Technology Inc, Stat Fax 3200) as a function of time. A standard curve of absorbance against the amount of naphthol produced was constructed to enable calculation of the amount of naphthol produced during esterase assay. The kinetic parameters were determined from Lineweaver–Burk plots. All measurements were conducted in triplicate.

GST: Adult mites were homogenized in ice-cold 0.2M phosphate buffer (pH 7.0) (enzyme preparation was similar to that mentioned for esterase but without Triton X-100). Later, the homogenates were centrifuged at 10,000g for 12 minutes at 4°C and GST activity was measured using 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced GSH as substrates with slight modifications according to Habig *et al.* (1974) in 96-well microplates. For this assay, 15 μ l enzyme, 100 μ l CDNB (1.2 mM) and 100 μ l GSH (10 mM) were added to a microplate. Enzyme activity was determined by continuously monitoring the change in absorbance at 340 nm for 5 minutes at 25°C with a microplate reader (Awareness Technology Inc, Stat Fax® 3200). To determine kinetic parameters, different final concentrations of CDNB (0.01-5 mM) at fixed concentration of GSH (10 mM) were used and the K_m and V_{max} values were also estimated from Lineweaver–Burk plots. The results were calculated based on an extinction coefficient of 9.6 mM cm^{-1} at 340 nm.

AChE: Enzyme extracts were prepared according to Khajehali *et al.* (2010) with some modification. Briefly, 1,000 female mites were homogenized in 3ml Tris-HCl buffer (10 mM, PH= 7.5) containing 0.4%

Triton X-100 and 1M NaCl and incubated for 20 min on ice. After incubation, the homogenate was centrifuged at 10,000g for 15 minutes at 4°C. AChE activity and its kinetic parameters with three substrates (ATC, BTC and PTC) were measured according to the modified method of Ellman described by Ghadamyari *et al.* (2008b). The concentration of substrate was changed from 0.01 to 5 mM for kinetic parameters. The results were expressed based on the molar extinction coefficient of 13.6 mM cm^{-1} at 412 nm. Experiments were performed in triplicate. The K_m and V_{max} values were estimated from Lineweaver–Burk plots.

MFO Assay

Using a heme-peroxidase assay, the total amount of heme containing proteins was measured (Brogdon *et al.*, 1997; Enayati and Motevali Haghi, 2007). The values were compared with a standard curve of purified cytochrome C and were reported as equivalent units of cytochrome P₄₅₀/mg protein corrected for the known content of cytochrome C and P₄₅₀. The reaction mixture in each well of the microtitre plate contained 20 μ l of mite homogenate (50 female adults were homogenized in 400 μ l of 0.1 M phosphate buffer, pH 7.0), 80 μ l of 0.625 M potassium phosphate buffer pH 7.2, 200 μ l of 3, 3', 5, 5' tetramethyl benzidine (TMBZ) solution (0.01 g TMBZ dissolved in 5 ml methanol plus 15 ml of 0.25 M sodium acetate buffer pH 5.0) and 25 μ l of 3% hydrogen peroxide. The plates were incubated at room temperature for 2 hours and absorbance was measured at 450 nm as an endpoint in the plate reader.

Non-denaturing Gel Electrophoresis and Zymogram Analysis

EST: Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed by the method of Davis (1964) using 7.5% gel (w/v). After electrophoresis, the gel was incubated in

0.1M phosphate buffer, pH 7.0, containing α -NA and β -NA (0.02 % each) and 0.1 % Fast Blue RR salt at room temperature for 1 hour. Following electrophoresis, the gel was incubated in 0.1% (w/v) boric acid for 20 minutes. This was then decanted and replaced with 0.1 M phosphate buffer, pH 7.0, containing 0.02% (w/v) α -NA and 0.02% (w/v) β -NA as substrates. The gel was stained with 0.1% (w/v) Fast Blue RR salt at room temperature for one hour.

GST: Non-denaturing polyacrylamide gel electrophoresis was carried out by Davis method (1964) using 10% co-polymerized gel with 0.08 % starch. Following electrophoresis, the gel was incubated in 8 mg CDNB and 14 mg reduced glutathione dissolved in 20 ml phosphate buffer 0.1M, pH 6.8, for 1 hour and stained with Lugol solution (I_2 0.25% and KI 0.25%) until the appearance of clear zones in protein bands with GST activity against a dark background.

Inhibition Assay of AChE

For the calculation of median inhibition concentration (IC_{50}) of AChE, inhibition assays were conducted by the pre-incubating the enzyme with various concentrations of chlorpyrifos-oxon (10^{-5} -10 mM) for 10 minutes. Afterward, the ATC substrate solution was added to the mixture containing Tris-HCl buffer, pH 8.0 and DTNB. Residual activity was estimated by kinetically measuring at 412 nm by the standard assay method as described above. IC_{50} values were measured by probit analysis using the POLO-PC computer program (LeOra Software, 1987). All experiments were conducted with three replicates.

RESULTS

Identification of Mites

Mites were identified to the species level using morphological keys (Zhang, 2003) and also based on molecular analysis. The length of the amplified sequences was about 900

base pairs. All the sequences showed 99% identity with *T. urticae* (accession number: AJ316605) and > 90% with *T. urticae* (AJ316598). This similarity search based on COI showed that these three populations belonged to the *T. urticae*.

Chlorpyrifos Resistance Levels

The LC_{50} (95% CI) values of ISR, Yz and GUS2 populations were calculated as 71.5 (50.02-102), 3.98 (2.76-5.34) and 0.4 (0.31-0.52) mg AI l^{-1} , respectively. The ISR population with resistance ratio (RR) of 176.90 showed high resistance to chlorpyrifos, whereas moderate levels of resistance were observed in Yz population (RR= 9.78).

Synergistic Effect

The LC_{50} of chlorpyrifos in the presence of synergists decreased in all populations (Table 1). The synergistic results suggest that the metabolic mechanisms do not play an important role in resistance to chlorpyrifos.

Detoxification Enzymes Assay

Results of kinetic parameters and activity of EST, GST and MFO contents in three populations of *T. urticae* are summarized in Table 2. The highest specific activity and specificity constant (V_{max}/K_m) for all populations were achieved when α -NA was used as substrate. V_{max}/K_m value of the ISR population was 1.69- and 1.34-fold higher than Yz and GUS2 using α -NA substrate, respectively. GST activity in the ISR and Yz populations was higher than GUS2 population and their specificity constants were 1.49- and 1.32-fold greater than those in the GUS2 population, respectively. MFO contents of both ISR and Yz populations increased when compared to GUS2 population.

**Table 1.** Effect of Piperonyl Butoxide (PBO), Diethyl Maleate (DEM) and Triphenyl Phosphate (TPP) on chlorpyrifos resistance in *T. urticae* populations.

Populations	Synergists	N	LC ₅₀ (95% CI) ^a	Slope±SE	χ ² (df) ^b	SR (95% CI) ^c
ISR	without	250	71.5 (50.02-102.1)	3.51±0.13	2.53 (3)	-
	PBO	250	42.93 (19.31-79.5)	2.45±0.41	3.15 (4)	1.66 (0.8-3.7)
	DEM	250	29.43 (17.3-52.4)	3.1±0.51	0.67 (2)	2.55 (>1.7)
	TPP	248	24.8 (14.96-54.15)	3.12±0.21	1.56 (3)	2.88 (>2.1)
Y _z	without	250	3.98 (2.76-5.34)	2.05±0.34	0.409 (2)	-
	PBO	248	2.02 (1.28-2.4)	1.91±0.27	1.54 (4)	1.97 (1.8-3.2)
	DEM	249	1.53 (1.14-2.05)	1.65±0.38	3.9 (3)	2.41 (1.35-3.96)
	TPP	250	1.44 (0.9-1.74)	1.47±0.69	1.01 (2)	2.83 (2.04-3.88)
GUS2	without	250	0.4 (0.31-0.52)	2.12 ±0.37	2.82 (2)	-
	PBO	249	0.21 (0.13-0.25)	2.04±0.65	3.91 (3)	1.9 (1.47-5.03)
	DEM	250	0.19 (0.15-0.25)	1.87±0.12	2.11 (2)	2.18 (1.5-3.4)
	TPP	250	00.15 (0.12-0.23)	1.38±0.22	2.74 (3)	2.67 (2.09-4.7)

^a The LC₅₀ value are expressed as mg AI/l and their 95% confidence intervals (95% CI); ^b Values of χ², lower than (P ≤ 0.05) indicate a significant fit between the observed and expected regression lines,

^c SR, Synergistic Ratio= LC₅₀ of acaricide/ LC₅₀ of (synergist+acaricide).

AChE Activity and Its Kinetic Parameters

The results of AChE kinetic parameters and its activity are shown in Table 2. K_m values of both ISR and Y_z populations were higher than GUS2 population for all the substrates, while no significant difference was observed in the V_{max}/K_m among the populations. These results showed that increase in K_m in ISR population led to low affinity of the enzyme to the substrate without changing the reaction characteristics.

Zymogram Banding Patterns

EST: The EST activity of *T. urticae* populations were analyzed by native PAGE. After activity staining, at least nine bands were detected (Figure 1). EST band intensities for ISR population were the highest. The effects of TPP on esterase activity were also evaluated on PAGE. In presence of TPP, EST band intensities in all populations were decreased and EST2 and EST7 bands in GUS2 population and EST8 band in Y_z population were completely inhibited after treatment with TPP. As

depicted in Figure 1, EST band intensities for these two populations, especially for GUS2, were less than that in ISR population.

GST: After GST activity staining, three major isoforms of GST could be clearly observed in all populations (Figure 2). Two weak extra bands were observed in ISR population. As depicted in Figure 2, after treatment with DEM, band intensities decreased in all populations.

Determination of Chlorpyrifos-oxon IC₅₀

The inhibitory effect of chlorpyrifos-oxon on AChE activity is shown in Figure 3. In addition, the insensitivity ratios were estimated at 23.30 and 2.96 for ISR and Y_z populations, respectively. The results indicated that less sensitivity of the AChE to chlorpyrifos occurred in resistant population compared to the other two populations.

DISCUSSION

The warm humid conditions in the greenhouse are ideal for resurgence of *T. urticae* due to its short life cycle and high reproductive capacity; and its management

Table 2. Kinetic parameters and activity (mean \pm SE) of AChE and detoxification enzymes in ISR, Yz and GUS2 populations (All the assayed were performed triplicate).

Enzyme	Substrate	ISR				Yz				GUS2			
		SA ^a	K _m (mM)	V _{max} (mM/min)	V _{max} /K _m	SA	K _m (mM)	V _{max} (mM/min)	V _{max} /K _m	SA	K _m (mM)	V _{max} (mM/min)	V _{max} /K _m
EST ^b	α -NA ^e	85.83 ^{ab*}	0.038 ^a	3.75 ^a	98.68 ^a	57.38 ^b	0.049 ^b	3.60 ^b	73.46 ^b	34.12 ^c	0.055 ^c	3.20 ^c	58.18 ^c
		\pm 7.20	\pm 0.009	\pm 0.05	\pm 8.51	\pm 10.21	\pm 0.012	\pm 0.02	\pm 8.04	\pm 6.43	\pm 0.007	\pm 0.04	\pm 7.08
	β -NA	68.70 ^a	0.056 ^a	3.85 ^a	68.70 ^a	59.02 ^b	0.063 ^b	3.70 ^a	59.02 ^a	38.90 ^c	0.079 ^c	3.00 ^b	38.91 ^b
		\pm 6.13	\pm 0.008	\pm 0.04	\pm 5.05	\pm 4.34	\pm 0.011	\pm 0.25	\pm 2.76	\pm 5.55	\pm 0.009	\pm 0.38	\pm 5.56
	α -NB ^f	42.50 ^a	0.055 ^a	2.33 ^a	42.50 ^a	34.50 ^b	0.058 ^a	2.00 ^{ab}	34.50 ^b	29.82 ^b	0.063 ^a	1.88 ^b	29.82 ^b
	\pm 8.12	\pm 0.011	\pm 0.032	\pm 3.05	\pm 3.99	\pm 0.011	\pm 0.18	\pm 6.21	\pm 3.46	\pm 0.004	\pm 0.25	\pm 3.45	
GST ^c	α -NP ^g	44.08 ^a	0.059 ^a	2.16 ^a	44.08 ^a	39.25 ^b	0.064 ^a	2.12 ^{ab}	39.25 ^b	37.67 ^b	0.066 ^a	2.09 ^b	37.67 ^b
		\pm 5.02	\pm 0.007	\pm 0.07	\pm 2.42	\pm 8.02	\pm 0.007	\pm 0.32	\pm 2.89	\pm 9.1	\pm 0.009	\pm 0.19	\pm 2.06
	CDNB ^h	138.5 ^a	0.29 ^a	79.16 ^a	273 ^a	115.41 ^b	0.32 ^{ab}	77.44 ^b	242 ^b	98.20 ^c	0.39 ^b	71.25 ^c	182.6 ^c
MFO ^d		\pm 12.43	\pm 0.04	\pm 6.43	\pm 12.98	\pm 9.52	\pm 0.035	\pm 1.23	\pm 14.32	\pm 6.98	\pm 0.006	\pm 4.25	\pm 10.5
	content	0.0068 ^a				0.0065 ^a				0.0041 ^b			
		\pm 0.001				\pm 0.001				\pm 0.001			
AChE ⁱ	ATC ^j	5.72 ^{ab*}	0.050 ^a	0.29 ^a	5.80 ^a	5.28 ^{ab}	0.041 ^b	0.21 ^b	5.6 ^{ab}	4.56 ^b	0.036 ^c	0.20 ^b	5.5 ^b
		\pm 0.32	\pm 0.005	\pm 0.01	\pm 0.32	\pm 0.63	\pm 0.002	\pm 0.05	\pm 0.22	\pm 0.31	\pm 0.001	\pm 0.04	\pm 0.31
	BTC ^k	4.56 ^a	0.044 ^a	0.20 ^a	4.54 ^a	4.25 ^b	0.033 ^b	0.15 ^a	4.55 ^a	4.10 ^b	0.029 ^b	0.13 ^a	4.48 ^a
	\pm 0.17	\pm 0.002	\pm 0.007	\pm 0.43	\pm 0.67	\pm 0.03	\pm 0.005	\pm 0.09	\pm 0.34	\pm 0.009	\pm 0.002	\pm 0.24	
	PTC ^l	5.54 ^{ab}	0.056 ^a	0.18 ^a	3.20 ^a	5.43 ^a	0.051 ^{ab}	0.16 ^a	3.13 ^a	4.63 ^b	0.047 ^b	0.15 ^a	3.2 ^a
		\pm 0.01	\pm 0.003	\pm 0.004	\pm 0.50	\pm 0.61	\pm 0.007	\pm 0.003	\pm 0.32	\pm 0.71	\pm 0.010	\pm 0.004	\pm 0.15

* Different letters indicate that the specific activity of enzymes in different populations is significantly variable as determined by Tukey's test ($p < 0.05$).

^a Specific activity (nmol/min.mg protein), ^b esterases, ^c glutathione-S-transferase, ^d mixed function oxidase (Unit of cytochrome P450/mite), ^e naphthyl acetate, ^f naphthyl butyrate, ^g naphthyl propionate, ^h 1-chloro-2, 4-dinitrobenzene, ⁱ acetylcholinesterase, ^j Acetylthiocholine iodide, ^k S-butrylthiocholine iodide, ^l propionylthiocholine iodide,

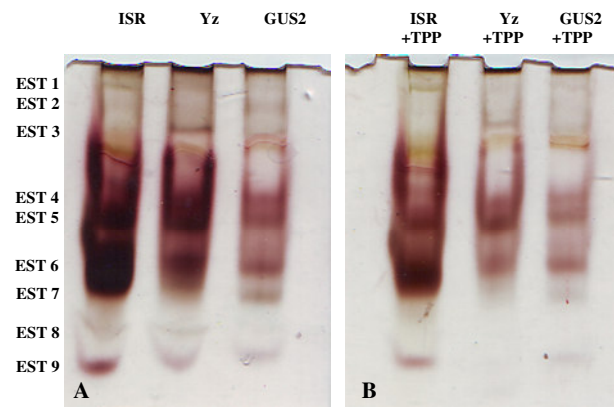


Figure 1. Zymogram of esterase (EST) in ISR, Yz and GUS2 populations. (A) Control and (B) Treated with Triphenyl Phosphate (TPP).

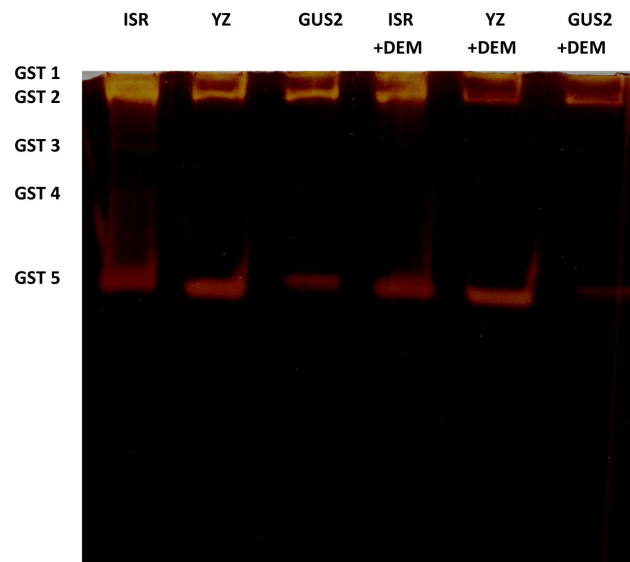


Figure 2. Glutathione-S-transferase (GST) banding pattern in ISR, Yz and GUS2 populations.

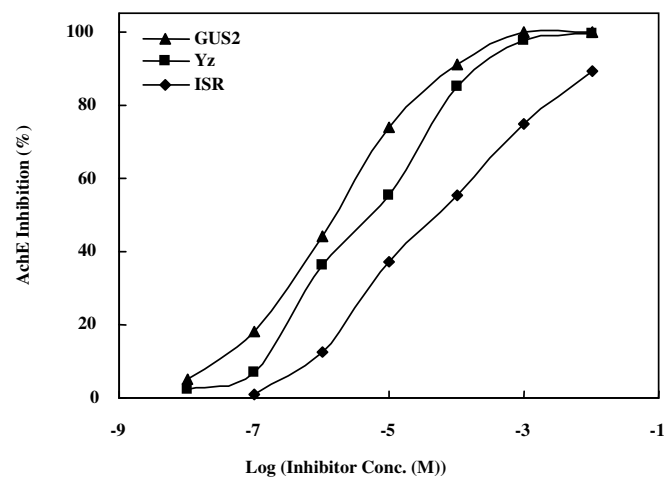


Figure 3. Inhibition of acetylcholinesterase (AChE) in *T.urticae* populations by chlorpyrifos-oxon.

is difficult in many agricultural systems due to rapid development of resistance to acaricides. The extensive and frequent use of these acaricides facilitates resistance development in some Iranian populations of *T. urticae* (Ghadamyari *et al.*, 2008; Memarizadeh *et al.*, 2011; Mahdavi Moghaddam *et al.*, 2012). Therefore, designing the program for management of acaricide resistance should be considered before loss of the chemical efficiency. In this study, resistance to chlorpyrifos was reported for the first time in Iranian populations of *T. urticae*. ISR and Yz populations showed high and moderate resistance to this acaricide, respectively, based on the LC₅₀ values (RR= 176.90 and 9.78). The LC₅₀ value obtained using RCV method in the present study was 0.4 mg AI/l for the susceptible population. Stumpf and Nauen (2001) calculated the LC₅₀ of chlorpyrifos on susceptible spider mite larvae by spray application as 4.1 mg technical acaricide/l. In Florida *T. urticae* collected from cotton displayed high resistance to this acaricide (RR= 78). Hellenic (ATHRos-Pm) and Belgium (MR-VL) *T. urticae* populations displayed resistance ratios of 78 and 586 against chlorpyrifos and Stumpf and Nauen (2001) calculated the LC₅₀ value of chlorpyrifos on susceptible strain (LS-VL) by Potter spray tower method as 11 mg formulated acaricide l⁻¹ (Khajehali *et al.*, 2010). Effects of metabolic enzymes (EST, MFO, and GST) on resistance mechanisms to chlorpyrifos were investigated by determining synergistic effects of TPP, PBO, and DEM, respectively. As depicted in Table 1, synergism effect caused a reduction in LC₅₀ values of this acaricide on all populations approximate equally. Therefore, these results showed that metabolic enzymes have no key role in resistance to chlorpyrifos. Detoxification enzymes are known to be involved in the metabolic resistance to acaricides (Ay and Gurkan, 2005; Van Leeuwen and Tirry, 2007). Determination of esterase and GST activity, their kinetic parameters, and zymogram analysis

indicated that ISR population had the highest specific activity and specificity constant among the populations. Also, the content of mixed function oxidases in ISR population was the highest. The enhanced activity of EST, GST, and content of MFO were probably due to resistance to other acaricides and not just chlorpyrifos. Therefore, synergistic results suggest that other mechanisms such as target site resistance should be involve in resistant to chlorpyrifos. To determine the role of AChE insensitivity in resistance mechanisms, kinetic parameters of this enzyme and inhibitory effect of chlorpyrifos-oxon as AChE inhibitor were investigated. Along with increasing the K_m value in Yz and ISR populations, V_{max} increased as well, so that no significant differences were observed in AChE specificity constant among the populations (Table 2). Also, the results showed that ISR and Yz populations were insensitive to chlorpyrifos-oxon compared with GUS2 population.

T. urticae was the first arthropod in which target site insensitivity was proven to be the resistance mechanism (Voss and Matsumura, 1964). Since then, biochemical studies have led to the conclusion that AChE insensitivity is the most common type of OP resistance in *T. urticae* (Van Leeuwen *et al.*, 2009). Insensitive AChE to OP acaricides is widespread and has been reported in *T. urticae* strains from Germany (Voss and Matsumura, 1964; Smislaert *et al.*, 1970), Japan (Anazawa *et al.*, 2003), and New Zealand (Ballantyne and Harrison, 1967) and in a few other tetranychid species including *T. cinnabarinus* from Israel (Zahavi and Tahori, 1970) and *T. kanzawai* from Japan (Kuwahara, 1982). The insensitivity of AChE to demeton-S-methyl, ethyl paraoxon, chlorpyrifos-oxon and carbofuran was also identified in a German laboratory strain of *T. urticae* and a field collected strain from Florida by Stumpf and Nauen (2001) who estimated AChE insensitivity ratios as 120 and 130 for VB and WI strains of *T. urticae*, respectively. Recently, Kwon *et al* (2010b) reported some



T. urticae AChE mutations that were associated with target site insensitivity in a Korean monocrotophos-resistant strain. In addition, the properties of each mutation were recently verified by functional expression (Kwon *et al.*, 2012). In Greece, insensitivity ratio using chlorpyrifos as inhibitor was obtained as 29 in ATHRos-Pm strain (Khajehali *et al.*, 2010). Molecular investigation suggest that amino acid substitution in the AChE catalytic center or near the active site may result in different responses of altered AChE to different substrate and inhibitors (Anazawa *et al.*, 2003; Khajehali *et al.*, 2010). So far, the reported mutation on AChE of *T. urticae*, collected from various countries, have differed from each other and different chlorpyrifos insensitivity for AChE in ISR and Yz populations may have different molecular mechanism as chlorpyrifos insensitivity. However, the amino acid sequences of AChE in these populations need to be analyzed.

In conclusion, the Iranian populations of two-spotted spider mite showed a good correlation between *in vivo* resistance to chlorpyrifos and insensitivity of their AChE to chlorpyrifos-oxon. Resistance and insensitivity ratios obtained were 176.90 and 23.3 for ISR and 9.78 and 2.96 for Yz population, respectively. Enhanced K_m values of AChE and less sensitivity to inhibition by chlorpyrifos-oxon in resistant population compared to the other populations suggest that resistant population had altered AChE target site. In other words, some amino acid substitutions and, subsequently, conformational changes occurred at the active site of the enzyme that reduced the AChE affinity to substrate and led to less sensitivity of enzyme to chlorpyrifos inhibitor.

ACKNOWLEDGEMENTS

The authors express their gratitude to the research council of the University of Guilan and Ministry of Sciences, Research, and

Technology for their financial support during the course of this project. We also thank Professor R. Sariri for her help.

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بررسی ساز و کارهای بیوشیمیایی مقاومت به کلرپایریفوس در کنه دو لکه ای
Tetranychus urticae (Acari: Tetranychidae)

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چکیده

در این مطالعه، ساز و کارهای بیوشیمیایی مقاومت به کلرپایریفوس در سه جمعیت کنه دو لکه ای برای اولین بار در ایران مورد بررسی قرار گرفته است. در ابتدا، سمیت سه جمعیت از کنه دو لکه ای که از استان های اصفهان (ISR)، یزد (Yz) و گیلان (GUS2) جمع آوری شدند به روش RCV تعیین شد و نسبت مقاومت در جمعیت های ISR (مقاوم) و YZ (نیمه مقاوم) در مقایسه با جمعیت GUS2 (حساس) به ترتیب ۱۷۶/۹ و ۹/۷۸ تخمین زده شد. تعیین فعالیت ویژه و پارامترهای سینتیکی آنزیم های استراز و گلوکاتایون اس- ترانسفراز نشان داد که جمعیت ISR بالاترین فعالیت و کارایی کاتالیتیکی را در بین جمعیت ها داراست. همچنین، مقدار MFO نیز در گونه مقاوم بیشتر از سایر جمعیت ها بود. هر چند که اثرات سینرژیستی PBO، DEM و TPP نشان داد که آنزیم های متابولیکی سهم عمده ای در مقاومت به کلرپایریفوس در جمعیت های ISR و YZ ندارند و افزایش در فعالیت استراز، گلوکاتایون اس- ترانسفراز و MFO احتمالاً به دلیل مقاومت به سموم دیگر می باشد. به منظور بررسی نقش غیرحساس شدن آنزیم استیل کولین استراز در مکانیسم مقاومت، پارامترهای سینتیکی استیل کولین استراز و اثر مهارتی-chlorpyrifos oxon روی این آنزیم، مورد مطالعه قرار گرفت Km. آنزیم با استفاده از سوبسترای استیل تیوکولین آیدواید، به ترتیب ۰/۰۳۶، ۰/۰۴۱، ۰/۰۵ میلی مولار برای جمعیت حساس، نیمه مقاوم و مقاوم تعیین شد. به علاوه، نسبت غیرحساس شدن استیل کولین استراز با سم کلرپایریفوس به ترتیب ۲۳/۳ و ۲/۹۶ برای جمعیت مقاوم و نیمه مقاوم تخمین زده شد. این نتایج، وجود جانشینی اسید آمینه ای را در جایگاه فعال و یا نزدیک به آن در این آنزیم تأیید می کند و نشان می دهد که جمعیت مقاوم دارای AChE تغییر یافته است.