RESISTANCE MECHANISMS TO OXYDEMETON-METHYL IN *TETRANYCHUS URTICAE* KOCH (ACARI: TETRANYCHIDAE)

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ABSTRACT: The resistance mechanisms to oxydemeton-methyl were surveyed in two Iranian strains of the two spotted spider mite, *Tetranychus urticae* Koch. Bioassay was carried out on two strains, collected from Tehran and Rasht using a dipping method. The results of bioassay indicated that resistance ratio was 20.47 for resistant strain. The activity of esterase and glutathione S transferase in resistant and susceptible strains showed that one of resistance mechanisms to oxydemeton-methyl was esterase-based resistance and glutathione S-transferase. The esterase activity of the resistant strain was 2.5 and 2.14-fold higher than those of the susceptible strain for α-naphtyl acetate (α-NA) and β-naphtyl acetate (β-NA) respectively. The kinetic characteristics acetylcholinesterase (AChE) showed that the AChE of resistant strain had lower affinity to artificial substrates; acetylthiocholine and butyrylthiocholine than that of susceptible strain. I50 of oxydemeton-methyl for resistant and susceptible strains were 2.68×10 M and 7.79×10 M respectively. The results suggested that AChE of resistant is insensitive to oxydemeton-methyl and ratio of AChE insensitivity of resistant to susceptible strain were 3.49 and 7.8-fold to oxydemeton-methyl and paraoxon, respectively.

KEY WORDS: *Tetranychus urticae*, Oxydemeton-methyl, Esterase, Insensitive acetylcholinesterase, Glutathione S-transferase

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is an important agricultural pest with a global distribution. Its phytophagous nature, high reproductive potential and short life cycle facilitate rapid resistance development to many acaricides often after a few applications (Cranham & Helle 1985; Keena & Granett, 1990; Devine et al., 2001; Stumpf & Nauen, 2001). So far resistance has been reported in several countries for compounds, such as organophosphates (OPs) (Sato et al., 1994; Anazawa et al., 2003), dicofol (Fergusson-Kolmes et al., 1991), organotins (Edge & James, 1986); hexythiazox (Herron & Rophail, 1993), clofentezine (Herron et al., 1993); fenpyroximate (Sato et al., 2004) and abamectin (Beers et al., 1998).

Insensitive AChE causing OP resistance is widespread and has been detected in *T. urticae* strains from Germany (Matsumura & Voss, 1964; Smissaert et al., 1970), Japan (Anazawa et al., 2003) and New Zealand (Ballantyne & Harrison, 1967) and in a few other tetranychid pest species, including *T. cinnabarinus* from Israel (Zahavi & tahori, 1970) and *T. kanzawai* from Japan (Kuwahara, 1982). Also the insensitivity of AChE to demeton-S-methyl, ethyl paraoxon, chlorpyrifos oxon and carbofuran was identified in a German laboratory strain of *T. urticae* and a field collected strain from Florida (Stumpf et al., 2001).

However, insensitive AChE was not the only mechanism of OP resistance in spider mites described, as some resistant strains of *T. urticae* showed an

enhanced degradation of malathion, malaoxon, and ethyl parathion to nontoxic products (Herne and Brown, 1969; Matsumura and Voss, 1964). OP-resistant strains of *T. kanzawai* rapidly degraded malathion *in vitro* and the resistance was obviously attributed to high nonspecific esterase activity (Kuwahara, 1981 and 1982). Pilz et al. (1978) showed that a German dimethoate-selected laboratory strain of *T. urticae* possessed multiple mechanisms of OP resistance. In addition to an AChE insensitive to dimethoxon, the toxicity of dimethoate was enhanced by synergists, such as piperonyl butoxide indicating the involvement of cytochrome P-450-mediated oxidative detoxication.

Oxydemeton-methyl is currently used in Iran to control some pests, such as aphids and *T. urticae* in several crops. The intensive use of oxydemeton-methyl to control of *T. urticae* and aphids in greenhouse facilitates resistance development in some populations of *T. urticae* in Iran. There is no information about oxydemeton-methyl resistance in this pest in Iran. Resolution of the underlying biochemical mechanisms of resistance can play an important role in circumventing problems associated with pesticide resistance and assist in rational choices of chemicals for pesticide mixtures and rotations. The purpose of this study was to collect information about the presence of esterases, gluthathion s-transferase and insensitive acetylcholinesterases in the resistance of *T. urticae* by bioassays and biochemical assays.

MATERIAL AND METHODS

Two spotted spider mite strains

The resistant strain was collected from infected been plants grown in the research greenhouse in Plant Pests and Disease Research Institute of Iran, Tehran. A strain from Rasht was considered as a strain susceptible to oxydemeton-methyl which had no previous exposure to pesticides and was collected from *Convolvulus sp.* in University of Guilan. The mites were reared routinely on bean plants (*Phaseolus vulgaris*) grown under greenhouse conditions (25 ± 4° C, 60 ± 20 RH).

Pesticide

Oxydemeton-methyl was used as the commercial formulation in the bioassay (EC 25%) and was purchased from Bayer Crop Science, Germany

Chemicals

Acetylthiocholine iodide (ATC), S-butyrylthiocholine iodide (BTC), 5,5'-dithiobis-(2-nitrobenzoic acid, DTNB), triton X-100 were purchased from Sigma. Fast blue RR salt, α-naphtyl acetate (α-NA) and β-naphthyl acetate (β-NA) were obtained from Fluka, and oxydemeton-methyl from Accustandard. 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) were purchased from Merck, Germany.

Bioassay

The toxicities of oxydemeton-methyl to the susceptible and resistant strains of

two-spotted spider mite were assayed using the dipping method. The formulated oxydemeton-methyl was diluted with distilled water to generate five serial dilutions. The leaf disk (diameter 3.5cm) was immersed in the dilutions for 45s. After drying, adult mites were placed on each treated leaf disk on wet cotton in a petri dish. Up to 10 adults were placed on each leaf disk. Mortality was assessed after the treated mites were maintained at 25 ± 2° C, 70 ± 10 RH and 16:8 (L:D) for 48h. Mites that could walk at least one body length after a gentle probe with a fine brush were scored alive. Bioassay data were analyzed for LD50 values and their 95% confidence intervals (95% CL) using the POLO-PC computer program (LeOra Software 1987). Resistance factors (RF) were calculated by dividing the LD50 value of the resistant strain by the LD50 value of the susceptible strain.

Determination of esterase activity

Adults were homogenized in ice-cold 0.2 M phosphate buffer (pH 7.0) containing 0.05 % triton X-100. After the homogenates were centrifuged at 10000 g for 12 min at 4°C. The esterase activity was measured according to van Asperen's method (van Asperen, 1962). The substrate was α-NA and β-NA. Fifteen µl of supernatant was added to a microplate containing 35 µl 0.2 M, pH 7.0, phosphate buffer per well. The addition of 100 µl substrate per well (0.65 mM in buffer) initiated a reaction. After incubation for exactly 10 min at room temperature, 50 µl of fast blue RR salt was added and the microplate left in the dark for 30 min. Absorbance at 450 nm (OD450) was then measured in a microplate reader (Awareness stat fax® 3200).

Determination of glutathione S-transferase (GST)

GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and reduced GSH as substrates with slight modifications according to Habig et al. (1974) in 96-well microplates. The total reaction volume per well of a 96-well microplate was 300 µl, consisting of 100 µl, supernatant, CDNB (or DCNB) and GSH in buffer, giving final concentrations of 0.4 and 4mM of CDNB (or DCNB) and GSH, respectively. The non-enzymatic reaction of CDNB (or DCNB) with GSH measured without supernatant served as control. The change in absorbance was measured continuously for 10 min at 340nm in a Thermomax kinetic microplate reader (Awareness stat fax® 3200).

AChE kinetics

Mites were homogenized in ice-cold 0.2 M phosphate buffer (pH 7.0) containing 0.1% triton X 100. After the homogenates were centrifuged at 10000 g for 15 min at 4 °C. AChE activity was measured according to the methods of Stumpf et al. (2001) with some modifications. Fifty microliters of the enzyme source was added to each well of microplate containing 140 µl of 0.2 M phosphate buffer (pH 7.0) and 20 µl DTNB solution. Then 40 µl of ATC was added to each well. The concentrations of the substrate were changed from 0.01 mM to 10 mM to evaluate the Michaels's constant (*Km*). Optical density was measured at 415 nm with a Microplate Reader (Awareness Stat fax® 3200).

Inhibition assay

The enzyme was preincubated with inhibitor at 37°C for 15 min. After preincubation, the ATC substrate was added to the mixture (containing 0.2 M phosphate buffer (pH 7.0) and DTNB). The remaining activity was determined at 30 min following preparation of the reaction mixture. Optical density was measured at 415 nm with a Microplate Reader (Awareness stat fax® 3200). I50 values for the AChE of susceptible and resistant strains were estimated by probit analysis using the POLO-PC computer program.

RESULTS

Resistance levels in bioassay

Table 1 summaries the toxicological data for susceptible and resistant strains exposed to oxydemeton-methyl. The resistance ratio of the resistant strain was 20.47.

Activity of esterase

The measured esterase activity of the resistant strain was significantly higher than that of the susceptible strain (t-test $P < 0.001$). The esterase activity of the resistant strain was 2.5 and 2.14- fold higher than those of the susceptible strain for α-NA and β-NA respectively (Fig. 1).

Activity of GST

The measured glutathione S-transferase activity of the resistant strain was significantly higher than that of the susceptible strain (t-test $P < 0.001$). The glutathione s-transferase activity of the resistant strain was 1.75 and 1.27-fold higher than those of the susceptible strain for CDNB and DCNB, respectively (Fig. 2).

Kinetic analysis of AChE.

The effect of substrate concentrations on AChE activity were investigated using ATC and BTC. The different specificities of AChE in resistant and susceptible strains toward two substrates are summarized in Table 2. ***Km*** values suggest that AChE in resistant strain was kinetically different from that in susceptible strain, indicating qualitative differences among enzymes in two strains. The kinetic study indicated that AChE from the resistant strain had 1.55 and 2.16-fold lower affinities to substrates ATC and BTC than the susceptible strain, respectively. AChE of the susceptible strain showed significantly higher affinity toward BTC than AChE of the resistant strain, suggesting that a modification of the enzyme catalytic site might be present in the AChE from the resistant mite.

Inhibition of AChE by oxydemeton-methyl and paraoxon

A comparison of the I50 values of the susceptible and resistant strains showed

3.49 and 7.8-fold resistance to oxydemeton-methyl and ethyl paraoxon, respectively (Table 3, Fig. 3).

DISCUSSION

Metabolic resistance mechanisms seem to be most important in arthropod species exhibiting resistance to organophosphate and carbamate pesticides (Ghadamyari, et al., 2008 a & b; Devonshire et al., 1982; Moores et al., 1994; Kono and Tomita, 1992). Our results showed that probably glutathione S-transferase was related to oxydemeton-methyl resistance in *T. urticae*, and there is 1.75-and 1.27-fold increase in glutathione S-transferase activity in the resistant strain, when CDNB and DCNB were used as substrate respectively. GSTs are detoxification enzymes frequently associated with insecticides resistance, particularly OP resistance (Soderlund and Bloomquist, 1990; Yu, 1996). These enzymes may act as binding proteins increasing the activity of other pesticide detoxification enzymes such as esterases (Grant and Matsumura, 1994).

Also esterases have a main role in resistance of *T. urticae* to oxydemeton-methyl (fig.1). These enzymes probably sequester or degrade insecticide esters before they reach their target sites in the nervous system. This mechanism seems to be important in the insecticide resistance of *Culex* mosquitoes (Mouches et al., 1986; Kono and Tomita, 1992; Tomita et al., 1996) and *Aphis gossypii* (Suzuki et al., 1993). The relationship between the enzymes which catalyze hydrolysis of β-NA and degredation of malathion was studied in resistance and susceptible strains of *T. kanzawai* Kishida by Kuwahara (1981). Their results showed that resistance to malathion was associated with increased esterase activity at E3 and E4 bonds on which the main peak of malathion degradation was detected. Although metabolic detoxification mechanisms are implicated, insensitive AChE is considered the principal mechanism of resistance to oxydemeton-methyl in *T. urticae*. The occurrence of pesticide-insensitive AChE in spider mite was first demonstrated by Smissaert (1964). The present study indicates that the resistant strain possesses an altered AChE with decreased sensitivity to inhibition by oxydemetn-methyl and paraoxon and decreased affinity to ATC and BTC substrates. The *Km* values for ATC determined in our study were 95 and 61 μM for the insensitive and sensitive forms of AChE, respectively (Table 2). Our results agree well with those reported by Anazawa et al. (2003) with respect to the involvement of insensitive AChE in conferring OP resistance in *T. urticae*. Because AChE from the resistant strain had reduced affinity to ATC and BTC (i.e. increased *Km* values) and reduced sensitivity to inhibition by oxydemetn-methyl and paraoxon (i.e. increased *I*50 values) compared with AChE from susceptible strain, it is clear that the resistant strain possesses qualitatively altered AChE. Recent molecular investigations suggest that some amino acid substitutions in the AChE of *T. urticae* may result in different responses of the altered AChEs to different substrates and inhibitors (Anazawa et al., 2003). At present the only biochemical tests available for monitoring insensitive AChE in the field based on inhibition assays (Bourguet et al., 1996). It will be difficult to develop for mites due to their minute size (Stumpf et al., 2001). Therefore the amino acid sequences of AChE in Iranian strains need to be analyzed.

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Table1. Log dose probit-mortality data for oxydemeton-methyl against susceptible and resistant strain of *T. urticae*

Strain	Insecticide	n	LD_{50} (95% CI)[a]	Slope ± SE	χ^2 [b]	RR[c]
Resistant	oxydemeton-methyl	245	4675.9 (4473- 4892)	10.79± 1.36	0.88	20.47
Susceptible	oxydemeton-methyl	250	228.6 (191-268)	2.5 ± 0.27	1.11	

[a]LD_{50} values and their CI are expressed in ppm formulated pesticide

[b]Values of χ^2 smaller than 7.81 ($p < 0.05$) considered to be represented satisfactory agreement between observed and expected results.

[c]Resistance ratio, LD_{50} of resistant strain/LD_{50} of susceptible strain

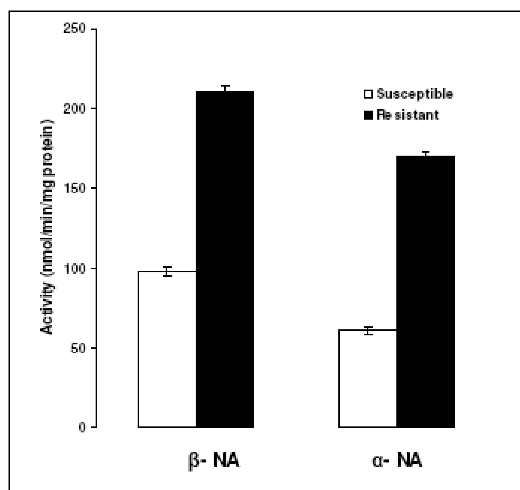

Fig.1. Esterase activity in resistant and susceptible strains of *T. urticae*

Table 2. *Km* and V_{max} values of AChE in resistant and susceptible strains of *T. urticae*.

Substrate	Strain	K_m (µM) (±SD)	V_{max} (ΔOD/30min/mite) (±SD)
ATC	resistant	95± 5.2	5 ± 0.4
	susceptible	61± 4.1	4.33 ± 0.31
BTC	resistant	337±32	3.2±0.27
	susceptible	156±23	2.9±0.23

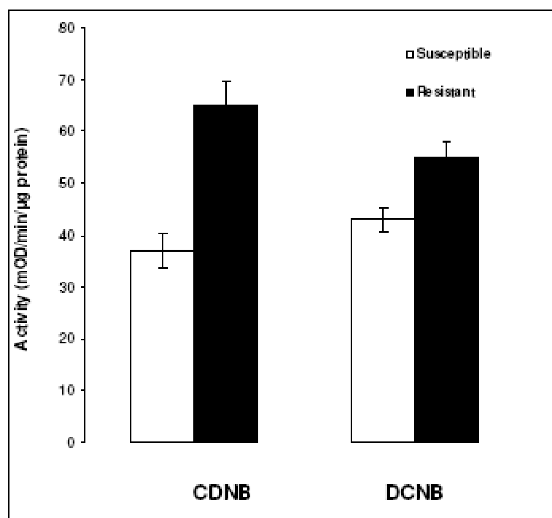

Fig.2. GST activity in resistant and susceptible strains of *T. urticae*

Table 3. I_{50} values of oxydemeton-methyl and paraoxon on AChE from susceptible and resistant strains of *T. urticae*

Inhibitor	I_{50} (M) (95%CI)		IR (95%CI)[a]
	Resistant	Susceptible	
Oxydemeton-methyl	2.68×10^{-6}	7.79×10^{-7}	3.49 (2.82-4.37)
	(2.3×10^{-6}- 3.15×10^{-6})	(6.6×10^{-7}- 9×10^{-7})	
Paraoxon	6.5×10^{-6}	8×10^{-7}	7.8(5.2- 11.8)
	(5.4×10^{-6}- 7.8×10^{-6})	(5.2×10^{-7}- 12.2×10^{-7})	

[a] Insensitivity ratio= I_{50} for resistant strain/susceptible strain and confidence interval (CI)

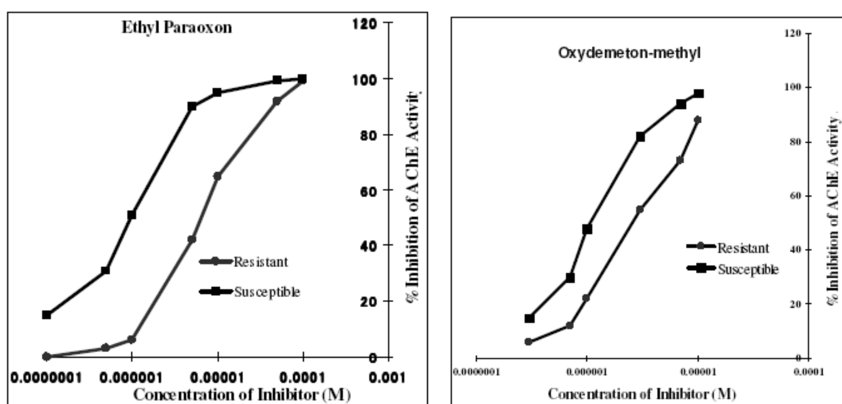

Fig. 3. Inhibition of AChE from *T. urticae* by oxydemeton-methyl and ethyl paraoxon