

RESISTANCE MECHANISMS TO ABAMECTIN IN IRANIAN POPULATIONS OF THE TWO-SPOTTED SPIDER MITE, *TETRANYCHUS URTICAE* KOCH (ACARI: TETRANYCHIDAE)

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ABSTRACT — The toxicity of abamectin to the Iranian populations of two-spotted spider mite, *Tetranychus urticae*, collected from Isfahan (ISR) and Guilan (GUS2) provinces were assayed using the residual contact vial (RCV) bioassay. The results interestingly showed ≥ 12755 -fold resistance to abamectin in the ISR population of *T. urticae* compared with the susceptible GUS2 population. The synergistic effects of Triphenyl Phosphate (TPP), Piperonyl Butoxide (PBO) and Diethyl Maleate (DEM) were carried out to determine the involvement of esterase, mixed functional oxidase (MFO) and glutathione S-transferase (GST) in resistance mechanisms, respectively. Due to very high levels of resistance in the ISR population, it was not possible to calculate LC₅₀ value nor to accurately assess the effects of synergists on this population. When α -naphthyl acetate (α -NA) was used as a substrate, the difference in esterase activity between the ISR and GUS2 populations was statistically significant, but low. Kinetic studies also indicated that the α -NA hydrolyzing esterases in the ISR population were different from those of the GUS2 population. Activity of GST in the ISR population was 2-fold more than that in the GUS2 population, and K_m and V_{max} values of the ISR population to a 1-chloro-2,4-dinitrobenzene (CDNB) substrate were 1.44 and 1.21 times lower and higher than those of the susceptible counterpart, respectively. The amount of heme content in the ISR population was 1.26 times more than that in the GUS2 population. Finally, comparing the nucleotide sequences of one of the transmembrane regions of the glutamate-gated chloride channel (GluCl1) from the two studied populations showed no substitutions in the deduced amino acid sequence of this region in the ISR population.

KEYWORDS — *Tetranychus urticae*; abamectin; resistance mechanisms; glutamate-gated chloride channel

INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), is an important agricultural pest and causes crop losses by direct feeding and reducing the photosynthetic rate in severe

infestations (Gorman *et al.* 2001). This pest show short life-cycle, high reproductive potential and arrhenotokous reproduction, which favor the development of resistance to acaricides very rapidly (Van Leeuwen *et al.* 2010).

Abamectin is a natural fermentation product of

the soil bacterium, *Streptomyces avermitilis* (Hayes and Laws 1995). Since 1998, abamectin has been the most widely used acaricide in Iran, but recently it has failed to provide satisfactory *T. urticae* control on some crops in fields and greenhouses (Memarizadeh *et al.*, 2011). Abamectin resistance in *T. urticae* was reported worldwide (Campos *et al.* 1995, 1996, Stumpf and Nauen 2002, Kwon *et al.* 2010a). Synergism studies by Stumpf and Nauen (2002) reported that in an abamectin-resistant strain of *T. urticae*, piperonyl butoxide (PBO) and diethyl maleate (DEM) had enhanced activities of mixed function oxidases (MFO) and glutathione S-transferases (GST). In addition to synergism studies, the involvement of GST and MFO in abamectin resistance was confirmed by direct measurement of the enzymatic activities of P₄₅₀s and GSTs, revealing a 13- and 11-fold increase, respectively (Stumpf and Nauen 2002). Recently, Kwon *et al.* (2010a) reported that MFO and esterase (Est) were associated with abamectin resistance in two resistant strains of *T. urticae* from South Korea. Investigation on the role of reduced penetration or adsorption and metabolic mechanisms in resistance to abamectin showed that resistant mites excreted more [3H]avermectin B1a after ingestion than their susceptible strain (Clark *et al.*, 1995). Clark *et al.* (1995) also detected higher oxidative breakdown followed by conjugation to glutathione, leading to an increased excretion. Cross-resistance between milbemectin and abamectin had also been observed by Sato *et al.* (2005) and might be linked to a common detoxification route. However, in contrast to the oxidative detoxification of abamectin proposed by Stumpf and Nauen (2002), resistance to milbemycins had been associated with increased esterase metabolism (Yamamoto and Nishida, 1981). In addition to metabolic resistance mechanisms (enhanced MFO, Est and GST activities) in the resistant strains of *T. urticae*, Kwon *et al.* (2010b) and Dermauw *et al.* (2012) have reported a point mutation on the GluCl1 in resistant *T. urticae*. Furthermore, a novel G326E mutation in GluCl3 is associated with high levels of abamectin resistance (Dermauw *et al.*, 2012). Previous researches on the resistant and susceptible insects and nematodes have shown that several target sites in the nervous system such

as the GluCl, γ -amino butyric acid (GABA)-gated chloride channel (GABR) and the histamine-gated chloride channel (HisCl) were implicated in resistance to avermectin and ivermectin (McCavera *et al.*, 2007).

Knowledge concerning the mechanisms of spider mite resistance to acaricides can play crucial role in circumventing problems associated with acaricide resistance and designing strategies to avoid resistance (Yang *et al.* 2002). In the study, the development of resistance in a *T. urticae* population was followed during repeated exposure to two acaricides (*i.e.* abamectin and fenazaquin). The objectives of this research were to determine metabolic resistance mechanisms to abamectin and study the effects of synergists for testing possible mechanisms involved in resistance. The relevant gene region of GluCl1, as a target site, was also amplified and sequenced in the resistant and susceptible populations of *T. urticae*.

MATERIALS AND METHODS

Mite populations

The resistant population (ISR) was collected from infested roses grown in a greenhouse in Isfahan, Iran. It has been sprayed with abamectin and fenazaquin for several generations prior to collection. A susceptible population (GUS2) was collected in an unsprayed area on *Vigna unguiculata* (L.) in the Guilan province near the city of Rasht. The mite populations were identified using combined morphological and molecular approaches (Mendonça *et al.*, 2011). Mites were morphologically identified using the key proposed by Zhang (2003). The sequences of COI fragment that were obtained were then cross-referenced with *T. urticae* accessions in the GenBank database to confirm morphological diagnostics. The Genbank accession numbers are HQ732265 and HQ732266 for ISR and GUS2 populations, respectively. The mites were reproduced routinely on cowpea plants (*V. unguiculata*) grown in plastic containers under greenhouse conditions (25 ± 3 °C, 60 ± 10 RH).

Pesticides

Abamectin (technical grade, 95 % pure) used in bioassay and biochemical assay was obtained from the Research Center of Pesticide and Fertilizer, Iran.

Chemicals

Triton X-100, Bovine Serum Albumin (BSA), Reduced Glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), PBO, DEM, TPP, Ammonium Persulfate (APS), Bromophenol blue, Tris, Acrylamide, N,N'-methylene-bisacrylamide (Bis-acrylamide), Tetramethylethylenediamine (TEMED), Phenol, Chloroform, Ethanol, Sodium Dodecyl Sulfate (SDS), α -Naphthyl Acetate (α -NA) and β -Naphthyl Acetate (β -NA) were purchased from Merck (Merck, Darmstadt, Germany). Fast blue RR salt was obtained from Fluka (Fluka, Buchs, Switzerland). 3', 5, 5' tetramethyl benzidine (TMBZ) were purchased from Panreac and Polymerase Chain Reaction (PCR) reagents were obtained from Bioneer (Bioneer, UK).

Bioassay

Determining median lethal concentration (LC₅₀)

The toxicity of abamectin to the GUS2 and ISR populations of two-spotted spider mite was assayed using the residual contact vial (RCV) bioassay (Kwon *et al.* 2010a). For estimating LC₅₀ values, abamectin was dissolved in acetone to various concentrations (0.112 – 100,000 ppm) and 4 replicates were used per concentration (5 concentrations for GUS2 population). Acetone was used as the control. 150 μ l of abamectin solutions were transferred to 5 ml glass vials (7 \times 1.1 cm), and the inside wall of the vials were coated with the abamectin solutions by rolling it under a fume hood for 1 h. After the vials had dried, 10-15 adult female mites (0-24 h old) per replicate were transferred into each abamectin-coated vials. The treated mites were maintained at 25 \pm 1 $^{\circ}$ C, 70 \pm 10 R.H. and mortality was assessed 10 h later. The criterion for death was that a mite should not move its appendages when prodded with a camel's hair brush. For each concentration, the observed mortality was corrected by Abbott's formula (Abbott, 1925). The estimated LC₅₀

values and the 95 % confidence limits were calculated from probit regressions using the POLO-PC computer program (LeOra, 1987) based on Finney (1971).

Determining synergistic effects of PBO, DEM and TPP

Evaluation of the synergistic effects of TPP, PBO and DEM was performed to determine the involvement of esterase, MFO and GST in resistance mechanisms, respectively. For determination of synergistic effects, glass vials were coated with 150 μ l of a dilution of PBO (a MFO inhibitor) or TPP (an esterase inhibitor) or DEM (a GST inhibitor) in acetone together with various concentrations of abamectin solution (7 concentrations for TPP and DEM, 6 concentrations for PBO). Four replicates were used per concentration and each synergist was then used as controls. Bracketing tests showed that a concentration of synergists of 200 ppm had a low level of toxicity (mortality less than 10 %), thus it was used in the experiments. The bioassays were conducted by RCV method, as described above.

Biochemical assays

Determining esterase activity and its kinetic parameters

Esterase activity was determined with the use of Van Asperen (1962) method. α -NA and β -NA were used as substrates. Fifty female adults were homogenized in 500 μ l of 0.1 M phosphate buffer, pH 7.0 containing 0.05 % (v/v) Triton X-100 on ice. Once homogenized, they were centrifuged at 10,000 \times g for 15 min at 4 $^{\circ}$ C. 12.5 μ l of supernatant were then added to a microplate containing 112.5 μ l phosphate buffer (pH 7.0) per well. After 3 min, 50 μ l of 1.8 mM substrate solution was added per well, initiating a reaction. Following the addition of 50 μ l of the fast blue RR salt, absorbance at 450 and 540 nm were measured in a microplate reader (Awareness Technology Inc, Stat Fax 3200) for α -NA and β -NA, respectively. The formation of the α -naphthol- and β -naphthol-fast blue RR dye complex was converted to a specific activity using standard curves, which were obtained from different concentrations of α -naphthol and β -naphthol

mixed with fast blue RR salt (0.075 %), respectively (Miller and Karn, 1980). Catalytic activities of the enzymes were determined at different concentrations of the substrates in the range of 0.1 to 6.4 mM in phosphate buffer at room temperature ($25 \pm 2^\circ\text{C}$) as kinetic assay. Michaelis-Menten constant (K_m) and maximal velocity (V_{\max}) were estimated from the Lineweaver-Burk plots.

Determining GST activity and its kinetic parameters

GST assays were conducted following the method of Habig *et al.* (1974) using CDNB as a substrate. For this assay 15 μl crude extract (enzyme preparation was similar to that previously mentioned for esterase, however without Triton X-100), 100 μl of 1.2 mM CDNB and 100 μl of 10 mM GSH were added to the microplate. Enzyme activity was determined by continuously monitoring the change in absorbance at 340 nm for 5 min at 25°C with a microplate reader (Awareness Technology Inc, Stat Fax 3200). Catalytic activities of the enzymes were determined at different concentrations of CDNB in the range of 0.05 to 1.6 mM and fixed concentration of GSH (10 mM), as described above. Kinetic parameters (K_m and V_{\max}) for this enzyme were also estimated from the Lineweaver-Burk plots.

Non-denaturing polyacrylamide gel electrophoresis and zymogram analyses

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out using a running gel 10 % (w/v) co-polymerized with 0.08 % starch for GST by the method of Davis (1964). Electrophoresis was performed with 100 V at 4°C , for histochemical staining of GST; the gel was incubated in 8 mg CDNB and 14 mg reduced glutathione dissolved in 20 ml phosphate buffer (0.1 M, pH 6.5) at room temperature with gentle agitation. Subsequently, the gel was subjected to staining with Lugol solution (I_2 0.25 % and KI 0.25 %) at an ambient temperature until the appearance of a clear zone in protein bands with GST activity against a dark blue background occurred.

Heme peroxidase assay

This assay measured the total amount of heme containing protein using a heme-peroxidase assay (Brogdon *et al.* 1997, Enayati and Motevalli Haghi 2007). The values were compared with a standard curve of purified cytochrome C and were reported as equivalent units of cytochrome P_{450} /mg protein corrected for the known content of cytochrome C and P_{450} . The reaction mixture in each well of the microplate contained 20 μl of mite homogenate (50 female adults were homogenized in 400 μl of 0.1 M phosphate buffer, pH 7.0), 80 μl of 0.625 M potassium phosphate buffer pH 7.2, 200 μl 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 μl of 3% hydrogen peroxide. Subsequently, the plates were incubated at room temperature for 2 h and the absorbance was measured at 450 nm as an endpoint in the plate reader.

PCR amplification of GluCl and sequencing

Genomic DNA was extracted from approximately 15 female mites per population by modified phenol-chloroform extraction method (Sambrook and Russell, 2001).

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 5 min, 34 cycles of denaturation at 93°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The specific primers used for amplification and sequencing of a 522-bp fragment of GluCl1, one of the transmembrane regions of the glutamate-gated chloride channel gene (GluCl1), are TuG1uF1 (5'- TGTGCCCTGTTG-TATGTTGG - 3') and TuG1uR1 (5'- AAAATGGC-GAAAAGGAAAGG -3'). The PCR products were recovered from the agarose gels (1 % (W/V)) then purified using a PCR Purification Kit (Bioneer, UK). The purified PCR products were sequenced in both directions by Biotech (USA).

Similar searches were carried out using BLAST N and BLAST P (Altschul *et al.* 1997) through the

TABLE 1: Effect of abamectin, PBO, DEM and TPP on abamectin resistant ISR and susceptible GUS2 populations of *T. urticae*.

Populations	Synergists	N ^a	LC ₅₀ (95 % CI) ^b	Slope ± SE	χ ² (df)	SR (95 % CI) ^c	MP ^d	MPR ^e
ISR	without	350	>100000 ^f	-	-	-	4.7	-
	PBO	300	ND ^g	-	-	-	40	8.51
	DEM	300	ND	-	-	-	31.91	6.8
	TPP	300	ND	-	-	-	36.84	7.83
GUS2	without	399	7.84 (3.69-41.65)	0.61 ± 0.16	1.246 (3)	-	-	-
	PBO	295	1.38 (0.55-12.13)	0.61 ± 0.18	1.74 (4)	5.63 (2.12-13.24)	-	-
	DEM	301	2.22 (0.81-25.79)	0.63 ± 0.18	4.10 (5)	3.84 (1.45-8.47)	-	-
	TPP	288	1.67 (0.60-25.57)	0.60 ± 0.19	1.87 (5)	4.94 (1.80-11.64)	-	-

^a Number of mites tested^b The LC₅₀ value are expressed as part per million (ppm) and their 95% confidence intervals (95 % CI).^c SR, Synergistic ratio= LC₅₀ of acaricide/ LC₅₀ of (synergist + acaricide).^d MP, mortality percentage of ISR population when treated with the highest useable concentration of abamectin (100,000 ppm) with and without synergists.^e MPR, Mortality percentage ratio= mortality percentage of (synergist + acaricide)/ mortality percentage of acaricide^f 50 % lethality unobtainable due to limited solubility.^g ND, not determined

NCBI server. Nucleotide and amino acid sequences of GluCl1 were derived from the EMBL/GenBank (Benson *et al.* 2004) and SwissProt (Bairoch and Apweiler 1999) databases, respectively. The multiple sequence alignment was performed with the CLUSTAL W program (Thompson *et al.* 1994).

Determining protein concentration

Protein concentration was estimated by the Bradford (1976) method, using bovine serum albumin as a standard.

Statistical analysis

Three or four replicates were conducted for all the biochemical assays and the means were compared by t-student's test. Statistical analyses were performed at $P = 0.05$ using the SAS software (SAS Institute, 2001).

RESULTS

Resistance level in bioassay

The LC₅₀ values of abamectin for two populations of *T. urticae* are given in Table 1. The highest concentration of abamectin that we could apply (100,000 ppm) resulted in a mortality of 4.7 % in the ISR

population, so it was impossible to calculate its LC₅₀. However, this population showed an extremely high degree of resistance to abamectin: estimated resistance factor $\geq 12,755$ (estimate produced in the POLO-PC output).

The effect of synergists on abamectin resistance

It was not possible to accurately assess the synergistic effects of synergists, since it was impossible to compare ISR LC₅₀ obtained both with and without synergists. Thus, no accurate information on the involvement of detoxification enzymes was obtained (Table 1). Mortality percentage of the ISR population treated with the highest concentration of abamectin (100,000 ppm) together with TPP, PBO and DEM and the LC₅₀ of the GUS2 population treated with these synergists are shown in table 1.

Esterase activity

Esterase activities in both populations are presented in Table 2. Esterase activity in the GUS2 resistant population was 1.18 and 1.02 fold greater than in the ISR susceptible strain when α -NA and β -NA are used, respectively. The difference was significant in the esterase activities between the two populations, only when α -NA was used as substrate ($t = 7.54$, $df = 4$, $P < 0.0017$).

TABLE 2: Comparison of Esterase and GST activity and kinetic parameters in the GUS2 and ISR populations of *T. urticae* in the presence of α -NA, β -NA, and CDNB.

	Esterase				GST	
	α -NA		β -NA		CDNB	
	ISR	GUS2	ISR	GUS2	ISR	GUS2
Activity ^a	165.54 \pm 1.47*	139.63 \pm 3.1	42.37 \pm 1.3	41.16 \pm 1.78	117.59 \pm 10.72**	58.79 \pm 3.09
K_m (mM)	0.095 \pm 0.002*	0.105 \pm 0.001	0.034 \pm 0.002	0.041 \pm 0.001	0.088 \pm 0.007*	0.127 \pm 0.006
V_{max} (mM/min)	39.23 \pm 0.79**	29.10 \pm 0.76	7.92 \pm 0.33*	6.34 \pm 0.18	68.28 \pm 1.57*	56.02 \pm 2.33
V_{max}/K_m	409.59 \pm 1.33**	274.99 \pm 6.84	231.14 \pm 3.98*	152.45 \pm 2.51	781.51 \pm 58.67**	438.33 \pm 3.19

^a Activity was expressed as nmol naphthol.min⁻¹.mg protein⁻¹ and nmol conjugated product.min⁻¹.mg protein⁻¹ \pm SE for esterase and GST, respectively.

One asterisk (*) and two asterisks (**) denotes that the values are significantly different at 0.05 and 0.01, respectively.

Substrate specificity of esterase

Kinetic studies indicated that the α -NA hydrolyzing esterase in the ISR *T. urticae* population was different from those of the GUS2 population in response to the changes of the substrate concentration (Table 2). However, there was no significant difference in the K_m value between the ISR and GUS2 populations using β -NA substrate while there was a significant increase in V_{max} value in the ISR population compared to that of the GUS2 population ($t = 4.21$, $df = 4$, $P < 0.0136$ for β -NA). As shown in Table 2, the ratio V_{max}/K_m (specificity constant) for both α -NA and β -NA was higher for ISR population when compared to its susceptible counterpart ($t = 19.29$, $df = 4$, $P < 0.0001$ for α -NA and $t = 16.73$, $df = 4$, $P < 0.0001$ for β -NA). In addition, the highest specificity constant for ISR and GUS2 populations was achieved when α -NA was used as the substrate ($t = 9.11$, $df = 4$, $P < 0.0008$ for V_{max} and $t = 4.01$, $df = 4$, $P < 0.0160$ for K_m).

GST activity and its kinetic parameters

GST activity in the ISR population was 2-fold greater than that in the GUS2 population when CDNB was used as the substrate (Table 2) ($t = 16$, $df = 4$, $P < 0.0001$). Kinetic studies indicated that GST catalyzing the formation of glutathione-conjugated adducts in the ISR population were different from those of the GUS2 population in response to the changes of the substrate concentrations (Table 2). The results showed that there was a significant increase (1.21-fold) in V_{max} and decrease (1.44-fold) in

K_m value in the ISR population compared to that of the GUS2 population (Table 2) ($t = 4.35$, $df = 4$, $P < 0.0122$ for V_{max} and $t = 3.88$, $df = 4$, $P < 0.0178$ for K_m). Furthermore, the ratio V_{max}/K_m (specificity constant) for CDNB was 1.78-fold higher for ISR population compared with susceptible GUS2 ($t = 5.84$, $df = 4$, $P < 0.0043$).

GST banding pattern

After GST activity staining, three major isoforms of GST could be clearly observed in the populations with different electrophoretic patterns. As depicted in Fig. 1, the intensity of GST2 isozyme in the ISR population was stronger than that in susceptible counterpart and GST1 was inhibited by abamectin in both populations.

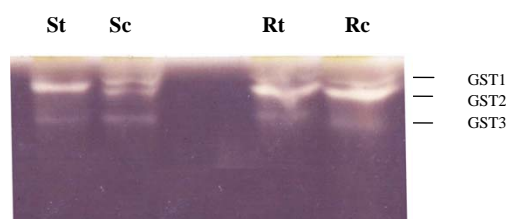
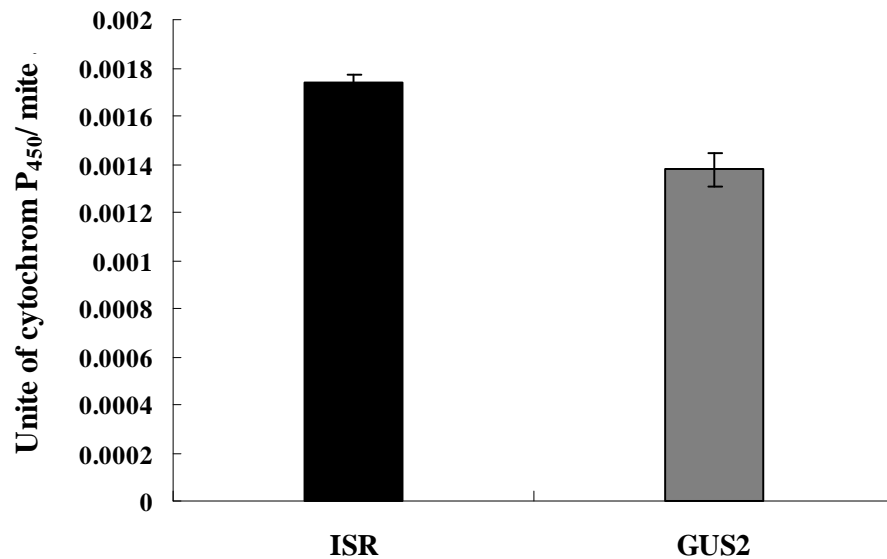


FIGURE 1: GST banding pattern in the susceptible GUS2 and resistant ISR populations of *T. urticae*. St: GUS2 mites treated with 1 ppm abamectin solution (using leaf dip method); Sc: GUS2 mites, control; Rt: ISR mites treated with 3000 ppm abamectin solution (using leaf dip method); Rc: ISR mites, control.

FIGURE 2: Mean \pm SE of heme content in the resistant ISR and susceptible GUS2 *T. urticae* populations.

TuGluCl_AbaS	MLVIVSWVSFWIDPNSAAARVLLGVTSLLTMSRQISGINASLPPVSYTKAVDIWTGCCLI
JX898521	MLVIVSWVSFWIDPNSAAARVLLGVTSLLTMSRQISGINASLPPVSYTKAVDIWTGCCLI
JX898522	MLVIVSWVSFWIDPNSAAARVLLGVTSLLTMSRQISGINASLPPVSYTKAVDIWTGCCLI
JQ738191	MLVIVSWVSFWIDPNSAAARVLLGVTSLLTMSRQISGINASLPPVSYTKAVDIWDCCLI
TuGluCl_AbaR	MLVIVSWVSFWIDPNSAAARVLLGVTSLLTMSRQISGINASLPPVSYTKAVDIWDCCLI

TuGluCl_AbaS	FVFGALIEFAIVNYVSRTDTIKADKHHRRRPQGIVGARRKFDTAKDSGIESSDVEDGPIG
JX898521	FVFGALIEFAIVNYVSRTDTIKADKHHRRRPQGIVGARRKFDTAKDSGIESSDVEDGPIG
JX898522	FVFGALIEFAIVNYVSRTDTIKADKHHRRRPQGIVGARRKFDTAKDSGIESSDVEDGPIG
JQ738191	FVFGALIEFAIVNYVSRTDTIKADKHHRRRPQGIVGARRKFDTAKDSGIESSDVEDGPIG
TuGluCl_AbaR	FVFGALIEFAIVNYVSRTDTIKADKHHRRRPQGIVGARRKFDTAKDSGIESSDVEDGPIG

TuGluCl_AbaS	YAKALGSTKLPRKKPPNRGIFSNWLSRFHTRSCKIDVTSR
JX898521	YAKALGSTKLPRKKPPNRGIFSNWLSRFHTRSCKIDVTSR
JX898522	YAKALGSTKLPRKKPPNRGIFSNWLSRFHTRSCKIDVTSR
JQ738191	YAKALGSTKLPRKKPPNRGIFSNWLSRFHTRSCKIDVTSR
TuGluCl_AbaR	YAKALGSTKLPRKKPPNRGIFSNWLSRFHTRSCKIDVTSR

FIGURE 3: Multiple sequence alignment of deduced amino acid sequences of one of the transmembrane regions of the *T. urticae* glutamate-gated chloride channel (GluCl1). Similar residues are shown with an asterisk and the position that differs from previously reported resistant strains is identified inside the box. [JX898521; resistant ISR population, JX898522; susceptible GUS2 population, JQ738191; resistant strain of *T. urticae* (Dermauw *et al.*, 2012), TuGluCl_AbaS and TuGluCl_AbaR; abamectin susceptible and resistant strains of *T. urticae*, respectively (Kwon *et al.*, 2010)].

Monoxygenases contents

The results of this assay, measuring the total amount of heme containing protein using a heme-peroxidase assay, indicated that the heme contents in the ISR population was 1.26-fold higher than that in the GUS2 population (Fig. 2) ($t = 7.38$, $df = 4$, $P < 0.0018$).

Sequence analysis of GluCl1

The amino acid sequences of *T. urticae*'s GluCl1 (TuGluCl1) was deduced from the nucleotide sequences for ISR and GUS2 populations. The amino acid sequence alignment of the sequences indicated that amino acid sequences of GluCl1 for these two populations were the same. The expected abamectin resistance mutation, G323D, reported by Kwon *et al.* (2010b), or G314 (equivalent with G323D) reported by Dermauw *et al.* (2012) in the TuGluCl1 or other mutations were not detected in the resistant population (Fig. 3). The sequences were submitted to GenBank with the following accession numbers: JX898521 and JX898522 for ISR and GUS2, respectively.

DISCUSSION

High resistance to abamectin in *T. urticae* has previously been demonstrated, 342-fold (Sato *et al.*, 2005), 1132 and 4753-fold (Kwon *et al.*, 2010a) and ≥ 3000 -fold (Memarizadeh *et al.* 2011). The estimated LC_{50} value obtained using RCV method in the present study was 7.87 ppm for the susceptible population, and more than 100,000 ppm for ISR population, showing the highest level of resistance to abamectin in *T. urticae* (more than 12,755-fold).

Oxidative, esteratic along with GST metabolism have now been implicated in abamectin resistance in a number of insects and mite species. Due to a very high level of resistance in the ISR population, it was not possible to calculate LC_{50} value and it was impossible to accurately assess the synergistic effects of synergists (Table 1). Although no synergistic effects were assessed, nevertheless this does not mean that detoxification enzymes were not involved in the resistance mechanism. Campos *et*

al. (1996) determined the effect of synergists on abamectin resistance. They reported relatively low synergistic ratios for PBO (between 0.7 and 2.7) and although abamectin could be synergized 7.9-fold by DEF in one population, inconsistent patterns were observed for all strains. Abamectin resistance in a Dutch strain was strongly synergized by PBO (4.4-fold) and DEM (6.1-fold) (Stumpf and Nauen 2002). Similar results were obtained in an abamectin resistant strain from Columbia (Stumpf and Nauen 2002). Lin *et al.* (2009) revealed that after application of PBO, DEM and TPP, the abamectin resistance ratio decreased from 8.6 to 5, 7.1 and 5.1, respectively.

In the study, investigating biochemical mechanisms of resistance to abamectin in the two-spotted spider mite indicated that the ISR population presented 1.18-fold higher esterase activity than the GUS2 population, when α -NA was used as a substrate. Overproduction and qualitative changes in enzyme structure are two general mechanisms by which esterases are associated with resistance to insecticides (Cao *et al.* 2008). Some esterases associated with resistance show lower catalytic efficiency toward insecticides but they are expressed in large quantities that effectively bind to insecticides, thus reducing their concentration in target sites (Field *et al.* 1988). Results showed that the ISR population presented a slightly higher affinity (*i.e.* 1.1-fold lower K_m value) to the substrate (α -NA) than the GUS2 population, suggesting that there was a low qualitative difference in α -NA-hydrolyzing esterases. Additionally, the V_{max} values in the ISR population were 1.35-fold higher than that of the GUS2 population (Table 2). A significant increase of the V_{max} value in the ISR population suggested that the α -NA-hydrolyzing esterases in the ISR population were also quantitatively different from those in the GUS2 population; consequently, the significant change of K_m and V_{max} values may suggest that the activity change of esterase is slightly quantitative and to a lesser extent qualitative.

The role of GSTs in the resistance to abamectin has been confirmed in *T. urticae* (Stumpf and Nauen 2002). In the present study, GSTs activity of the ISR population was significantly higher than that of the GUS2 population, which demonstrated that

the development of resistance to abamectin in *T. urticae* was correlated with the increase of GSTs activity. The results of the kinetic studies demonstrated that K_m and V_{max} values of GST to the substrate CDNB in the ISR population were significantly different from that of the GUS2 population; 1.44 and 1.21 times lower and higher than that of the GUS2 population. This suggested that GST catalytic efficiency and substrate affinity was enhanced after the development of resistance to abamectin in *T. urticae*. The GST activity was also characterized by zymogram analysis. The active band for one isoenzyme of the GST (*i.e.* GST2) was much stronger in the ISR population than in the GUS2 population. Furthermore, GST1 was inhibited by abamectin in the ISR and GUS2 populations, when leaf dip was used as a treatment method. Meng *et al.* (2002) showed that the increase of specific activity of GSTs was one of the main resistance mechanisms of *Panonychus citri* McGregor to fenpropathrin. Fan and Cheng (1996) reported that the dominant mechanism of *T. truncatus* to dicofol was the enhancement in GST. Lin *et al.* (2009) also demonstrated that GST was involved in resistance of *T. cinnabarinus* Boisduval to abamectin. Therefore, the role of GSTs has certain universality in the spider mites' resistance mechanism. However, Kwon *et al.* (2010a) indicated no obvious role of GST in abamectin resistance.

Mixed function oxidases (MFO) play a central role in the insecticide metabolism (Gong 1983). All preceding studies have confirmed the important role of MFO on the mechanisms of resistance to abamectin on the insects and mites (Argentine *et al.* 1992, Stumpf and Nauen 2002, Lin *et al.* 2009, Kwon *et al.* 2010a). However, the results of the present study indicated that the heme contents in the ISR population were only 1.26-fold higher than that in the GUS2 population. Van Leeuwen *et al.* (2006) showed that the amount of heme peroxidase activity in the resistant strain of *T. urticae* to chlorfenapyr was 2-fold lower than that of susceptible counterpart. They hypothesized that decrease in 3,3',5,5'-tetramethylbenzidine (TMBZ) peroxidase activity in the resistant strain could reflect decreasing activation of chlorfenapyr. Enayati and Motevalli Haghi (2007) demonstrated that the heme contents

in pyrethroid resistant strains of the German cockroach were 4.6 and 1.58-fold higher than that in a susceptible strain. Furthermore, the results of Moghadam *et al.* (2011) showed that the amount of heme contents in two resistant population of *T. urticae* to fenazaquin was 1.8 and 1.9 fold higher than susceptible population. In *P. citri*, the enhancement of relative activity of carboxylesterase, acetylcholinesterase (AChE), MFO and GST was reported as the major mechanism of resistance to fenpropathrin, while the resistance to abamectin was caused mainly by an increased activity of MFO and AChE (Meng *et al.* 2002).

The molecular mechanisms of abamectin resistance were investigated by Kwon *et al.* (2010b), revealed no polymorphism in cDNA fragments of GABAR genes in the susceptible and resistant populations. However, sequence comparison of the full-length cDNA fragment of a TuGluCl1 identified a G323D point mutation at one of the transmembrane regions of the GluCl1 in the abamectin resistant strain (Kwon *et al.*, 2010b). A mutation at this position confers dramatic insensitivity to avermectin in the vertebrate glycine receptor (Lynagh and Lynch, 2010). Results of the present study, by sequence comparison of the genomic DNA fragment of a GluCl1, showed that the expected abamectin resistance mutation, G323D, in the *T. urticae* GluCl1, reported by Kwon *et al.* (2010b), was not detected in ISR population. Khajehali *et al.* (2011), using the same sequence comparison, reported that although ten out of 15 strains under investigation in their study displayed abamectin resistance and four strains were resistant to both abamectin and milbemectin, the reported G323D mutation was not detected in these strains. Recently Dermauw *et al.* (2012) identified six orthologous GluCl genes (GluCl1, GluCl2, GluCl3, GluCl4, GluCl5 and GluCl6) in the genome of *T. urticae*. Furthermore given the involvement of GluCl channels in abamectin resistance, sequence comparison of GluCl1, GluCl2, GluCl3, GluCl4, GluCl5 and GluCl6 genes in abamectin resistant and susceptible strain revealed a novel G326E resistance mutation in GluCl3 that is associated with high levels of abamectin resistance (Dermauw *et al.* 2012). In ad-

dition to GluCl, the GABR and the HisCl are known to be a target site of avermectin and ivermectin in insects and nematodes (McCavera *et al.*, 2007).

In conclusion, slightly elevated esterase and GSTs activities and the low increasing heme contents in the ISR population showed a low level of involvement of the metabolic resistance mechanisms to abamectin. They cannot be responsible for the high level of abamectin resistance detected (more than 12,755-fold). This suggests the involvement of other resistance mechanisms, such as insensitivity of target site. According to the results of Dermauw *et al.* (2012), McCavera *et al.* (2007) and the present study, other GluCl, the GABR, or the HisCl could be involved in the resistance of ISR population. Therefore, the amino acid sequences of other possible target sites such as GluCl2, GluCl3, GluCl4, GluCl5, GluCl6, GABR and HisCl genes in ISR and GUS2 populations require further investigation and analyses.

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