Article

Comparison of susceptibility of two Iranian populations of *Tetranychus urticae* Koch (Acari: Tetranychidae) to spirodiclofen

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**ABSTRACT**

Two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae), is one of the most important agricultural pests throughout the world. The high reproductive potential, short life cycle, combined with frequent acaricide applications has resulted in resistance development to a wide range of acaricides. Resistance to acaricides has an important role in inefficiency of chemical control of this pest. In the present study, the susceptibility of two populations of two-spotted spider mite collected from Karaj (KrS) and Mahallat (MhR) to spirodiclofen was investigated. The bioassay was conducted using a leaf-dip method on same-age protonymphs. Bioassay results showed that there was a significant difference between LC$_{50}$ values of KrS and MhR populations. The resistance ratio was obtained as 22.19. The synergistic effects of triphenyl phosphate (TPP), piperonyl butoxide (PBO) and diethyl maleate (DEM) were assessed using residual contact vial (RCV) bioassay method. Results of the synergistic studies showed significant differences between LC$_{50}$ values of spirodiclofen with spirodiclofen + PBO and spirodiclofen + TPP in MhR population. The most synergistic effect in MhR population was related to PBO. The results of enzyme assays revealed that the most ratio activity in MhR population to KrS population was related to cytochrome P450 monoxygenase (3.02) and the lowest ratio activity was related to glutathione S-transferase (GST) (1.40). These results confirmed that esterase and cytochrome P450 monoxygenase are probably involved in resistance of *T. urticae* to spirodiclofen.

**KEY WORDS:** Detoxification enzymes; resistance mechanisms; spirodiclofen; synergists; two-spotted spider mite.

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**INTRODUCTION**

*Tetranychus urticae* Koch (Acari: Tetranychidae) is one of the most important pests of crops and garden plants around the world. This species is very polyphagous and about 1200 plant species belonging to 250 families of vegetables, fruit trees and ornamental plants are the hosts of this pest. This pest is mainly located on the underside of the leaves and feeds on the plant sap producing silk webbing that is clearly visible at high infestation levels. Signs of its feeding are the appearance of white-tailed yellowish spots on the upper surface of the leaf, which occurs due to depletion of the cells from chlorophyll. Finally, the leaves become yellow and tan. Severe infestation leads to burning, leaf fall and even plant death (Jeppson *et al.* 1975; Migeon and Dorkeld 2010).

There are various methods for controlling two-spotted spider mite including cultural, biological and chemical control. Moist and cool conditions as a cultural method decline the spider mite development and decrease feeding and reproduction. The biological control of this species is mainly based on the protection of natural enemies and release of Phytoseiid predator mites (Opit *et al.*...
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Although some progress has been made in controlling this species, the main control method of this species is still chemical control (Van Leeuwen et al. 2004). *Tetanychus urticae* has a high potential to become resistant to pesticides, due to its short life cycle, the high number of generations and parthenogenesis; as a result nowadays this species shows resistance to almost all groups of synthetic pesticides (Stumpf and Nauen 2002). Based on the Arthropod Pesticide Resistance Database (APRD), resistance of this pest to 95 pesticides in 501 cases has been reported, including resistance to abamectin (Memarizadeh et al. 2011; Ferreira et al. 2015), fenpyroximate (Kim et al. 2004), spiromesifen (Sato et al. 2016), chlorpyrifos (Zamani et al. 2014; Farahani et al. 2016) and propargite (Mohammadzadeh et al. 2014; Farahani et al. 2016). There are several mechanisms for the emergence and development of pesticide resistance including 1. Decreased cuticular penetration, 2. Increase in excretion, 3. Metabolic detoxification that is carried out through detoxification enzymes, 4. Target-site insensitivity. Among these mechanisms, metabolic detoxification and target site insensitivity are the most important causes of resistance development.

One of the pesticide groups recently developed to control mites and sucker pests is tetronic acid derivatives. Spirodiclofen, a derivative of these compounds, was introduced in 2002 to control the important economic mites belonging to *Tetranychus*, *Panonychus*, *Brevipalpus*, *Phyllocoptes* and *Aculus* species, and is now used as one of the major pesticides. Spirodiclofen is a selective and non-systemic compound, which prevents biosynthesis of fats (Nauen et al. 2003; Bretschneider et al. 2007). Due to different effects of the derivatives of tetronic acid, spirodiclofen has no cross-resistance with other pesticide groups, including organo-phosphorus compounds, mitochondrial electron transport inhibitors, hexithiazox and abamectin (Wachendorff et al. 2002; Konanz and Nauen 2004; Pree et al. 2005; Van Leeuwen et al. 2005). This compound is mainly effective on eggs and all stages of growth of spider mites, but it has only limited acute toxicity on mature stages, however, it is effective in the reproduction of adult female mites (Nauen et al. 2003; Bretschneider et al. 2007). Although spirodiclofen has been used for more than a decade, there are few documented reports on resistance to spirodiclofen (Van Pottelberge et al. 2009; Demaeght et al. 2013).

Despite the extensive use of spirodiclofen in Iran, there are no documented reports on the status of *T. urticae* regarding its resistance to this acaricide and the probable mechanisms involved. Therefore, the aim of present study was the determination of the status of two-spotted spider mite resistance collected from Mahallat’s ornamental greenhouses and the probable mechanisms at play through synergistic assay and biochemical study.

**MATERIALS AND METHODS**

*Populations*

A resistant population was collected from infested ornamental greenhouses in Mahallat (Markazi Province, Iran) which had a history of resistance to a number of acaricides including abamectin and propargite (Mohammadzadeh et al. 2014). A susceptible population (KrS) was collected from acarology laboratory of the College of Agriculture and Natural Resources, University of Tehran, which had never been exposed to any pesticides. Two populations were reared separately on potted kidney bean plants, *Phaseolus vulgaris* L. (Fabaceae) in a growth chamber (26 ± 1 °C, 60 ± 5% RH and 16L: 8D photoperiod) (Mohammadzadeh et al. 2014).

*Bioassay test*

To provide same age mites for using in the experiments, about 20 adult females were transferred to the upper side of 9 cm² square-cut kidney bean leaf discs on wet cotton wool and permitted to lay eggs for 12h. The plates were then placed in a growth chamber at 26 ± 0.5 °C, 60% RH and 16:8 h light: dark photoperiod. The toxicity of spirodiclofen (SC 24%, BAYER) to the KrS and MhR populations of *T. urticae* was studied using leaf-dip bioassay method (Tirello et al. 2012).
Based on preliminary tests, five concentrations were determined as the final concentrations causing 10 to 90% mortality. Experiments were performed in three replications. Distilled water was used in the control. The mortality rate after the emergence of adult female mites in the control was estimated using the following equation:

\[ \%M = \frac{(T - A)}{T} \times 100 \]

M is the percent of mortality; T is the total number of 1st instar nymphs and A is the number of appeared mature female mites. The resistance ratio was calculated by dividing the LC\textsubscript{50} of resistant population by the sensitive population LC\textsubscript{50} (Van Pottelberge et al. 2009).

**Synergism studies**

In order to determine the role of each detoxifying enzyme in developing resistance, inhibitors of these enzyme systems (including Piperonyl Butoxide (PBO), Diethyl maleate (DEM) and Triphenyl phosphate (TPP)) were used through the residual contact vial (RCV) bioassay (Van Leeuwen et al. 2004). Bracketing tests were performed to determine safe dose of each synergist. The highest dose of each synergist that caused less than 10% mortality was selected for final bioassay (Alizadeh et al. 2011). Final concentrations of synergists (PBO 210 mg mL\textsuperscript{-1}, DEM 540 mg mL\textsuperscript{-1} and TPP 1000 mg mL\textsuperscript{-1}) were used in bioassays.

Protonymphs were treated for two hours at a final concentration of each synergist and then treated with spirodiclofen. The experiment was carried out at five concentrations and three replications. Twenty protonymphs were used in each concentration. The mortality rate was determined as described in the bioassay method with spirodiclofen (Van Pottelberge et al. 2009). The synergistic factor was calculated from the LC\textsubscript{50} ratio of spirodiclofen without synergist to LC\textsubscript{50} of spirodiclofen treatment with synergist (Kalyanasundaram and Das 1985).

**Biochemical tests**

**Protein content**

Protein content was determined by Bradford method (1976) and using bovine serum albumin as the standard protein.

**Enzyme preparation**

Preparation of glutathione S-transferase (GSTs) and cytochrome monoxygenase P450 (P450) enzyme sample was performed by homogenizing 100 female T. urticae in cold buffer sodium phosphate (0.1 M and pH 7) on ice using a Potter-Elvehjem homogenizer, while the source of esterases was prepared by homogenizing 50 female mites in chilled buffer sodium phosphate (0.1 M and pH 7) containing 0.1% (w/v) Triton X-100. The homogenized samples were then centrifuged at 10,000 g for 15 min and the supernatant was used for measurement of enzyme activities (Kwon et al. 2010).

**Determination of GST activity**

The activity of GSTs was quantified according to the method of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) as substrates. The reaction mixture in wells of a 96-well micro-plate consisted of 15 µl supernatant, 50 µl CDNB (63 mM), 100 µl GSH (10 mM), and 150 µl phosphate buffer (0.1 M, pH 7.5). Enzyme activity was determined by the change in absorbance as measured every 30 s for 5 min at 340 nm using micro-plate reader (ELX808 Bio-Tek).

**Mono-oxygenase P450 contents**
The activity of P450 enzymes was measured according to the method of Brogdon et al. (1997) using 3,3,5,5′-tetraethyl benzidine (TMBZ) as substrate. The reaction mixture, containing 20 µl supernatant, 80 µl phosphate buffer (0.625 M, pH 7.2), 200 µl TMBZ, and 25 µl H2O2 (3%) per well of a 96-well micro-plate, was incubated at room temperature for 2 h, after which the absorbance was measured at 450 nm as an end-point in the plate reader. A standard curve of absorbance against the amount of purified cytochrome C was constructed to calculate the equivalent units (EU) of cytochrome P450 per milligram of protein.

Esterase activity measurement

Esterase activity was measured according to van Asperen (1962) method using α-naphthyl acetate (α-Na) as substrate. The reaction mixture, containing 20 µl supernatant, 70 µl phosphate buffer (0.1 M, pH 7), and 90 µl substrate (30 mM in acetone) per well of a 96-well microplate, was incubated at room temperature for 30 min followed by the addition of 90 µl fast blue RR. The absorbance was read at 450 nm for α-Na and at 540 nm for β-Na every 2 min for 20 min using a microplate reader (ELX808 Bio-Tek).

All biochemical tests were performed in three replications and in the control treatment, instead of the enzyme, the reaction buffer was used.

Data analysis

Lethal concentrations of 50% and 95% confidence intervals were estimated using the POLO-Plus software. The data on enzyme activities between the two populations were subjected to Student's t-test. All statistical tests were performed using SPSS statistical software version 22.

RESULTS AND DISCUSSION

LC50 values for spirpidiclofen in two populations of T. urticae calculated from probit analysis are given in Table 1. Significant differences (p < 0.05) were observed between the LC50 values of the populations. The LC50 value in Karaj and Mahallat were estimated 9.48 and 210.39 mg L⁻¹, respectively. Bioassay results proved that KrS population as the susceptible population. The resistance ratio in Mahallat population to KrS population was 22.19 (the LC50 value of Mahallat population to KrS population) (Table 1). Dose-response slope in Karaj and Mahallat populations were 2.75 ± 0.36 and 2.60 ± 0.32, respectively (Table 1). Seyed-Talebi et al. (2014) reported that LC50 values of spirodiclofen against larvae and protonymphs of two-spotted spider mite population were 4.81 and 4.04 mg mL⁻¹, respectively. Demaeght et al. (2013) assessed susceptibility of three populations of T. urticae to spirodiclofen. They recorded that LC50 values of the susceptible and resistant population were 7.4 and > 5000 mg mL⁻¹.

Today many farmers complain about the efficacy of acaricides used against T. urticae (Mohammadzadeh et al. 2014). There are several reasons for inefficiency of pesticides in pest control including: inappropriate timing of pesticide application, incorrect spraying techniques, undesirable quality of pesticides and pest resistance to pesticides. So every failure in pest control should not be seen as developing resistance to pesticides (Edwards 1975; Rola and Pingali 1993). Obtained results proved that the main reason for failure in control of MhR population is resistance development. Failures in the chemical control of T. urticae in Mahallat County caused by resistance have been reported for different compounds including abamectin, propargite, chlorpyrifos (Mohammadzadeh et al. 2014; Farahani et al. 2016).

As mentioned above the calculated resistance ratio by Demaegh et al. (2013) was 680 while in our study this was 22.17. The reason of this remarkable difference is that they put the susceptible population under continuous pressure of spirodiclofen while the history of spirodiclofen use in Mahallat's ornamental greenhouses is short meaning this compound has only recently been used.
The synergistic effects of TPP, PBO and DEM were evaluated on Karaj and MhR populations of *T. urticae* to determine the involvement of esterase, MFO and GST detoxifying enzymes in resistance mechanisms, respectively. The bioassay results showed that the LC$_{50}$ values of spirodiclofen + PBO, spirodiclofen + DEM and spirodiclofen + TPP in KrS population were 6.06 (4.98–7.15), 9.81 (8.11–11.53), 8.33 (7.20–9.51), and in MhR population 65.63 (53.9–77.25), 183.1 (157.08–210.84) and 126.3 (106.64–146.72) mg L$^{-1}$, respectively (Table 1). Statistical analyses indicated significant differences between LC$_{50}$ values of spirodiclofen + PBO-treated and spirodiclofen-treated MhR populations and spirodiclofen + TPP-treated and spirodiclofen-treated MhR populations. The calculated synergist ratio showed that spirodiclofen was 3.21 times more toxic in the presence of PBO than in the absence of PBO. According to calculated synergistic ratio, PBO had the highest synergist effect in both populations. Van Pottelberge et al. (2009) stated that LC$_{50}$ values of spirodiclofen in two populations of two spotted spider mites were 4.90 and 1343 mg L$^{-1}$. They calculated the resistance ratio as 274. They also demonstrated that the synergistic ratio of PBO on spirodiclofen in susceptible and resistant populations were 1.2 and 3.5, respectively, and DEM had no synergistic effect on spirodiclofen. Rauch and Nauen (2002) revealed that ester cleavage and oxidative detoxification were the main degradation pathways of this pesticide in *T. urticae*.

### Table 1. The effect of PBO, DEM and TPP on resistance of two spotted spider mite to spirodiclofen in KrS and MhR populations.

<table>
<thead>
<tr>
<th>Population</th>
<th>mite</th>
<th>LC$_{50}$ (mg L$^{-1}$) CI 95%$^a$</th>
<th>$\chi^2$(df)</th>
<th>Slope ± SE</th>
<th>SR$^b$ CI 95%</th>
<th>RR$^c$ CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>KrS</td>
<td>360</td>
<td>9.48 (7.93–11.03)</td>
<td>3.3 (13)</td>
<td>2.75 ± 0.36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KrS + PBO</td>
<td>360</td>
<td>6.06 (4.98–7.15)</td>
<td>4.67 (13)</td>
<td>2.28 ± 0.31</td>
<td>1.49 (1.91–1.88)</td>
<td>-</td>
</tr>
<tr>
<td>KrS + DEM</td>
<td>360</td>
<td>9.81 (8.11–11.53)</td>
<td>2.53 (13)</td>
<td>2.61 ± 0.40</td>
<td>0.97 (0.76–1.22)</td>
<td>-</td>
</tr>
<tr>
<td>KrS + TPP</td>
<td>360</td>
<td>8.33 (7.20–9.51)</td>
<td>2.36 (13)</td>
<td>3.08 ± 0.38</td>
<td>1.14 (0.92–1.41)</td>
<td>-</td>
</tr>
<tr>
<td>MhR</td>
<td>360</td>
<td>210.39 (178.54–243.57)</td>
<td>2.93 (13)</td>
<td>2.60 ± 0.32</td>
<td>-</td>
<td>22.19 (17.75–27.74)</td>
</tr>
<tr>
<td>MhR + PBO</td>
<td>360</td>
<td>65.63 (53.9–77.25)</td>
<td>2.53 (13)</td>
<td>2.69 ± 0.37</td>
<td>3.21 (2.54–4.05)</td>
<td>-</td>
</tr>
<tr>
<td>MhR + DEM</td>
<td>360</td>
<td>183.1 (157.08–210.84)</td>
<td>4.17 (13)</td>
<td>2.72 ± 0.33</td>
<td>1.15 (0.93–1.42)</td>
<td>-</td>
</tr>
<tr>
<td>MhR+TPP</td>
<td>360</td>
<td>126.13 (106.64–146.72)</td>
<td>2.14 (13)</td>
<td>2.74 ± 0.33</td>
<td>1.67 (1.34–2.08)</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ Confidence interval.
$^b$ SR, Synergistic Ratio = LC$_{50}$ of acaricide/ LC$_{50}$ of (synergist + acaricide).
$^c$ RR, Resistance Ratio = LC$_{50}$ of MhR population/ LC$_{50}$ of KrS population.

Thus, pre-treatment of two-spotted spider mites with PBO and TPP separately for 2 hours and then treatment with spirodiclofen resulted in a significant reduction of LC$_{50}$ values compared to treatment with spirodiclofen without synergist, which showed clearly the involvement of cytochrome P450 monoxygenases and esterases in resistance of *T. urticae* to spirodiclofen. **Detoxification enzymes**
The activity of general esterases was measured with α-naphthyl acetate as substrate in two populations. The results showed that enzyme activity was 0.089 ± 0.002 and 0.17 ± 0.007 mmol min^{-1} mg protein^{-1} in Karaj and Mahallat populations, respectively (Table 2). Quantitative analysis of general esterase activity revealed that amount of esterase activity in MhR population was 1.91 fold higher than KrS population. Statistical analyses showed a significant difference in esterase activity between the two populations (p < 0.01) (Table 2). Zamani et al. (2014) reported that the carboxyl esterase activity was 34.12, 57.38, and 85.83 nmol min^{-1} mg protein^{-1} in Guilan, Yazd and Esfahan populations of T. urticae, respectively.

The level of GST activity was assessed to be 0.0565 ± 0.0011 and 0.0792 ± 0.0031 mmol min^{-1} mg protein^{-1} in two populations of Karaj and Mahallat, respectively. Data analyses demonstrated a significant difference in GST activity between the populations at 99% confidence level (P ≤ 0.01). The GST activity ratio was calculated by the division of the GST activity in resistant population and the GST activity in susceptible population which showed this enzyme's activity in resistant population was 1.40 fold higher than susceptible population (Table 2). Van Pottelberge et al. (2009) reported the GST activity ratio as 5.2. Rauch and Nauen (2002) maintained susceptible strain of T. urticae for 37 generations and 21 months under spirodiclofen pressure. They observed that the resistance factor increased 13 times and the activity level of carboxyl esterase, GST and cytochrome P450 monooxygenase also increased.

Measuring the total amount of heme-containing protein using a heme-peroxidase assay, indicated that the cytochrome P450 contents in Mahallat was 3.02 fold higher than in KrS population. Based on statistical analyses there was a significant difference between amount of this enzyme in two populations (P < 0.01) (Table 2). The amount of p450 was 0.061 ± 0.0082 and 0.184 ± 0.0064 mmol min^{-1} mg protein^{-1} in Karaj and MhR populations, respectively. Van Pottelberge et al. (2009) reported that monooxygenase activity in resistant population was 11 fold higher than susceptible one. They concluded that monooxygenase plays an important role in resistance to spirodiclofen in T. urticae. Functional characterization of cytochrome P450s in T. urticae has not kept pace with that in other pests. However, Demaeght et al. (2013) recently expressed and functionally characterized two cytochrome P450s, CYP392E7 and CYP392E10. They showed that CYP392E10 is responsible for metabolizing tetronic acid acaricides, including spirodiclofen and spiromesifen.

In conclusion, our results reveal that MhR population has become resistant to spirodiclofen confirming growers’ complaints about the inefficacy of spirodiclofen. Combined bioassay, biochemical and synergistic data indicate that metabolic resistance via esterase and cytochrome P450 monooxygenase are involved in spirodiclofen resistance.

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نویسنده مستند

چکیده

بررسی حساسیت دو جامعیت Tetranychus urticae (Acari: Tetranychidae) به سپرودیکلوفن

روش گونه‌وری دیسک‌های برجی در محلول سمی با استفاده از ماده فرمله شده سپرودیکلوفن (SC 24%) انجام گرفت. نتایج نشان داده که حساسیت جامعیت دو کرگدن در سطح جمعیتLC50 میزان درصد بود. نسبت میزان LC50 در دو جمعیت صفت دار میزان 5 درصد می‌باشد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد. نتایج کناره‌گیری از وجود تفاوت معنی‌دار در میزان LC50 دو جمعیت نشان داد.

واژگان کلیدی: آزمایش‌های سم‌زده، مکانیسم‌های مقاومت، سپرودیکلوفن، سپرودیکلوفن، کناره‌گیری، کرگدن، دو جمعیت