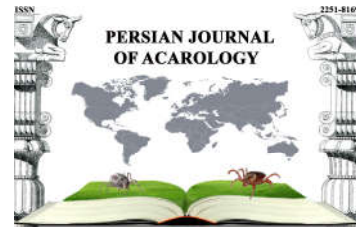




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## Article

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

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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 spiroadiclofen 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<sub>50</sub> 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<sub>50</sub> values of spiroadiclofen with spiroadiclofen + PBO and spiroadiclofen + 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 monooxygenase (3.02) and the lowest ratio activity was related to glutathione S-transferase (GST) (1.40). These results confirmed that esterase and cytochrome P450 monooxygenase are probably involved in resistance of *T. urticae* to spiroadiclofen.

**KEY WORDS:** Detoxification enzymes; resistance mechanisms; spiroadiclofen; 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.*

2001). 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). *Tetranychus 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 tetrionic acid derivatives. spiroadiclofen, a derivative of these compounds, was introduced in 2002 to control the important economic mites belonging to *Tetranychus*, *Panonychus*, *Brevipalpus*, *Phyllocoptruta* and *Aculus* species, and is now used as one of the major pesticides. spiroadiclofen 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 tetrionic acid, spiroadiclofen 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 spiroadiclofen has been used for more than a decade, there are few documented reports on resistance to spiroadiclofen (Van Pottelberge *et al.* 2009; Demaeght *et al.* 2013).

Despite the extensive use of spiroadiclofen 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 ( $26 \pm 1$  °C,  $60 \pm 5\%$  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 cm<sup>2</sup> 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  $26 \pm 0.5$  °C, 60% RH and 16:8 h light: dark photoperiod. The toxicity of spiroadiclofen (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 1<sup>st</sup> instar nymphs and A is the number of appeared mature female mites. The resistance ratio was calculated by dividing the LC<sub>50</sub> of resistant population by the sensitive population LC<sub>50</sub> (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<sup>-1</sup>, DEM 540 mg mL<sup>-1</sup> and TPP 1000 mg mL<sup>-1</sup>) were used in bioassays.

Protonymphs were treated for two hours at a final concentration of each synergist and then treated with spiroadiclofen. 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 spiroadiclofen (Van Pottelberge *et al.* 2009). The synergistic factor was calculated from the LC<sub>50</sub> ratio of spiroadiclofen without synergist to LC<sub>50</sub> of spiroadiclofen 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 monooxygenase 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  $\mu$ l supernatant, 80  $\mu$ l phosphate buffer (0.625 M, pH 7.2), 200  $\mu$ l TMBZ, and 25  $\mu$ l H<sub>2</sub>O<sub>2</sub> (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  $\alpha$ -naphthyl acetate ( $\alpha$ -Na) as substrate. The reaction mixture, containing 20  $\mu$ l supernatant, 70  $\mu$ l phosphate buffer (0.1 M, pH 7), and 90  $\mu$ l 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  $\mu$ l fast blue RR. The absorbance was read at 450 nm for  $\alpha$ -Na and at 540 nm for  $\beta$ -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

LC<sub>50</sub> values for spiridiclofen in two populations of *T. urticae* calculated from probit analysis are given in Table 1. Significant differences ( $p < 0.05$ ) were observed between the LC<sub>50</sub> values of the populations. The LC<sub>50</sub> value in Karaj and Mahallat were estimated 9.48 and 210.39 mg L<sup>-1</sup>, respectively. Bioassay results proved that KrS population as the susceptible population. The resistance ratio in Mahallat population to KrS population was 22.19 (the LC<sub>50</sub> value of Mahallat population to KrS population) (Table 1). Dose-response slope in Karaj and Mahallat populations were  $2.75 \pm 0.36$  and  $2.60 \pm 0.32$ , respectively (Table 1). Seyed-Talebi *et al.* (2014) reported that LC<sub>50</sub> values of spirodiclofen against larvae and protonymphs of two-spotted spider mite population were 4.81 and 4.04 mg mL<sup>-1</sup>, respectively. Demaeght *et al.* (2013) assessed susceptibility of three populations of *T. urticae* to spirodiclofen. They recorded that LC<sub>50</sub> values of the susceptible and resistant population were 7.4 and  $> 5000$  mg mL<sup>-1</sup>.

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 Demaeght *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.

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

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

<sup>a</sup> Confidence interval.

<sup>b</sup> SR, Synergistic Ratio = LC<sub>50</sub> of acaricide/ LC<sub>50</sub> of (synergist + acaricide).

<sup>c</sup> RR, Resistance Ratio = LC<sub>50</sub> of MhR population/ LC<sub>50</sub> of KrS population.

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<sub>50</sub> values of spiroidiclofen + PBO, spiroidiclofen + DEM and spiroidiclofen + 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<sup>-1</sup>, respectively (Table 1). Statistical analyses indicated significant differences between LC<sub>50</sub> values of spiroidiclofen + PBO-treated and spiroidiclofen-treated MhR populations and spiroidiclofen + TPP-treated and spiroidiclofen-treated MhR populations. The calculated synergist ratio showed that spiroidiclofen 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<sub>50</sub> values of spiroidiclofen in two populations of two spotted spider mites were 4.90 and 1343 mg L<sup>-1</sup>. They calculated the resistance ratio as 274. They also demonstrated that the synergistic ratio of PBO on spiroidiclofen in susceptible and resistant populations were 1.2 and 3.5, respectively, and DEM had no synergistic effect on spiroidiclofen. Rauch and Nauen (2002) revealed that ester cleavage and oxidative detoxification were the main degradation pathways of this pesticide in *T. urticae*.

**Table 2.** Activity (mean ± SE) of detoxification enzymes in KrS and MhR populations of *T. urticae*.

Enzyme	Substrates	Enzyme activity <sup>a</sup> ± SE		R/S <sup>b</sup>
		KrS	MhR	
Esterase	α-NA	0.089 ± 0.002	0.17 ± 0.007**	1.91
GST	CDNB	0.056 ± 0.0011	0.079 ± 0.003*	1.40
P450	TMBZ	0.061 ± 0.0082	0.184 ± 0.0064**	3.02

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

<sup>a</sup> Activity was expressed as mmol min<sup>-1</sup> mg protein<sup>-1</sup>.

<sup>b</sup> Enzyme activity in MhR population/enzyme activity in KrS population.

Thus, pre-treatment of two-spotted spider mites with PBO and TPP separately for 2 hours and then treatment with spiroidiclofen resulted in a significant reduction of LC<sub>50</sub> values compared to treatment with spiroidiclofen without synergist, which showed clearly the involvement of cytochrome P450 monooxygenases and esterases in resistance of *T. urticae* to spiroidiclofen.

#### Detoxification enzymes

The activity of general esterases was measured with  $\alpha$ -naphthyl acetate as substrate in two populations. The results showed that enzyme activity was  $0.089 \pm 0.002$  and  $0.17 \pm 0.007$  mmol min<sup>-1</sup> mg protein<sup>-1</sup> 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<sup>-1</sup> mg protein<sup>-1</sup> in Guilan, Yazd and Esfahan populations of *T. urticae*, respectively.

The level of GST activity was assessed to be  $0.0565 \pm 0.0011$  and  $0.0792 \pm 0.0031$  mmol min<sup>-1</sup> mg protein<sup>-1</sup> in two populations of Karaj and Mahallat, respectively. Data analyses demonstrated a significant difference in GST activity between the populations at 99% confidence level ( $P \leq 0.01$ ). 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  $0.061 \pm 0.0082$  and  $0.184 \pm 0.0064$  mmol min<sup>-1</sup> mg protein<sup>-1</sup> 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 tetroneic 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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## بررسی حساسیت دو جمعیت *Tetranychus urticae* Koch (Acari: Tetranychidae) به اسپیرودیكلوفن

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### چکیده

کنه تارتن دو لکه‌ای *Tetranychus urticae* (Acari: Tetranychidae) یکی از مهم‌ترین آفات محصولات کشاورزی در سراسر جهان است. پتانسیل زیاد تولید مثل، کوتاهی دوره زندگی، رژیم غذایی چندخواری همراه با کاربرد مکرر کنه‌کش‌ها، توانایی این گونه را در گسترش مقاومت به کنه‌کش‌ها افزایش داده است. مقاومت به کنه‌کش‌ها نقش اساسی در کارا نبودن کنترل شیمیایی این آفت ایفا می‌کند. در این پژوهش، حساسیت کنه تارتن دو لکه‌ای به اسپیرودیكلوفن در دو جمعیت جمع‌آوری شده از کرج و محلات مورد بررسی قرار گرفت. زیست‌سنجی به روش غوطه‌وری دیسک‌های برگگی در محلول سمی با استفاده از ماده فرموله شده اسپیرودیكلوفن (SC 24%) انجام شد. نتایج زیست‌سنجی حاکی از وجود تفاوت معنی‌دار در میزان LC<sub>50</sub> دو جمعیت در سطح اطمینان ۹۵ درصد بود. نسبت میزان LC<sub>50</sub> جمعیت محلات به جمعیت کرج ۲۲/۱۹ برآورد شد. مطالعات سینرژیستی با استفاده از سینرژیست‌های تری‌فنیل فسفات (TPP)، دی‌اتیل مالئات (DEM) و پایرونایل بوتوکساید (PBO) به روش لوله آزمایش انجام گرفت. نتایج مطالعات سینرژیستی تفاوت معنی‌دار در میزان LC<sub>50</sub> محاسبه شده در هنگام کاربرد سینرژیست‌های PBO و TPP را نشان داد. بیشترین تأثیر سینرژیستی در جمعیت محلات مربوط به سینرژیست PBO بوده است. براساس نتایج آزمون‌های بیوشیمیایی بیش‌ترین نسبت فعالیت آنزیمی در جمعیت محلات به کرج مربوط به آنزیم مونواکسیژناز P450 (برابر با ۳/۰۲) و کم‌ترین نسبت مربوط به آنزیم گلوکوتایون اس- ترانسفراز (برابر با ۱/۴۰) است. با توجه به نتایج به دست آمده از این مطالعه، می‌توان چنین نتیجه‌گیری کرد که آنزیم‌های استراز و مونواکسیژناز P450 احتمالاً در مقاومت *T. urticae* به اسپیرودیكلوفن دخیل هستند.

واژگان کلیدی: آنزیم‌های سم‌زدا؛ مکانیسم‌های مقاومت؛ اسپیرودیكلوفن؛ سینرژیست‌ها؛ کنه تارتن دو لکه‌ای.

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