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Research Article

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Study on the resistance to chlorpyrifos in *Microcerotermes diversus* (Isoptera: Termitidae)

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ABSTRACT- *Microcerotermes diversus* Silvestri, is an important termite in Khuzestan province with a broad range of foraging. In recent years, control of underground termites has been more based on the application of chemicals which has increased pest insecticide resistance. In this study, the resistance of different populations of termites to chlorpyrifos was investigated using estimated LC_{50} and measuring the activity of two acetylcholinesterase (AChE) and glutathione-s-transferase enzymes. Four populations of *M. diversus* with different histories of organophosphorus insecticide spraying were studied. Populations A (Am Altamir1), B (Am Altamir2), and C (Mollasani) were collected from date palm groves with 30 and 10-year histories of organophosphorus insecticide spraying, as well as without spraying history, respectively. Population D (Ramin University) was collected from orange trees, three weeks after spraying with chlorpyrifos. The bioassay results showed that the highest and lowest LC_{50} values were observed in populations A and C, respectively. Moreover, the activity of AChE using acetylthiocholine iodide, propionylthiocholine iodide, and butyrylthiocholine iodide substrates was higher in the populations with chlorpyrifos spraying history, with the highest activity in population A. It was also demonstrated that the AChE activity with acetylthiocholine iodide substrate was higher than the activity of this enzyme with butyrylthiocholine iodide and the propionyl choline substrates in the populations with 30 and 10-year history of spraying. The Michaelis constant (K_m) and maximum velocity (Vmax) values of AChE as two important kinetic factors, indicated the highest affinity of this enzyme to the substrate in population A.

INTRODUCTION

Termites are the most successful and important structural groups of insects on Earth that have colonized many habitats. They can injure annual and perennial crops and wooden components in dwellings (Verma et al., 2009). *Microcerotermes diversus* Silvestri (Isoptera: Termitidae) has become particularly problematic on palms in Khuzestan province, Iran, by causing serious economic damage to wooden products in buildings (Habibpour, 2010; Cheraghi, 2013). The termites feed within the trunks, roots, and leaves of palms, leading to small-diameter galleries throughout the palm accompanied by fine granular frass which are typical signs of termite damage (Behdad, 1984).

Effective termite control using conventional methods is often difficult and requires a significant volume of termiticide. On the other hand, chemical control has been widely used to reduce the infestation of termites and prevent their attack (Verma et al., 2009). Among these, organochlorine pesticides have been used routinely to control termites. However, the application of organochlorine pesticides has been banned due to their environmental hazards and risks to human health (Yeoh & Lee 2007). Chlorpyrifos has been introduced as a proper alternative insecticide widely used to control soil termites (Verma et al., 2009, Elango et al., 2012).

As continuous and excessive use of insecticides may result in resistance, monitoring of resistance to insecticides is necessary (Feyereisen, 1995). Upon exposure to chemical compounds, various species of insects use three main mechanisms including biochemical, physiological, and behavioral resistance. Probably, the most common type of insect resistance is biochemical resistance, in which the insecticides are detoxified, before reaching their target sites, by one or more enzymes (Whalon et al., 2008). Several researchers have reported that glutathione S-transferase (GSTs) and acetylcholinesterase (AChE) are enzymes involved in the detoxification of organophosphorus (OPs) insecticides (Wilce and Parker 1994; Zhu et al., 2011). GSTs are enzymes involved in detoxification of the harmful electrophilic endogenous and exogenous compounds including pesticides (Wilce & Parker 1994).

AChE is a key enzyme in the nervous system of both vertebrates and invertebrates, which terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. The enzyme is the primary target of inhibition by OP pesticides (Russell et al., 2004). Despite enormous reports regarding the biochemical resistance of insects, there isn't any study on the biochemical resistance of *M. diversus*.

Therefore, in the present study, the activity of two detoxifying enzymes, GST and AchE, was determined on various populations of *M. diversus* sprayed by chlorpyrifos insecticide

MATERIALS AND METHODS

Insects

Four termite populations were collected from three different locations with termite damages. Population A (Am Altamir1) was collected from palm groves of the Tropical Date in Tropical Research Institute, Ahvaz, Iran, directions 31°15'01.9"N 48°33'18.3"E, in early June 2017, 10 days after spraying. The population had been treated with various pesticides, including organophosporus (OPs) insecticides, such as diazinon, chlorpyrifos, and malathion, during the past 30 years. Population B (Am Altamir2) was collected from the same location in early June 2017 with a 10-year history of spraying. Population C was collected from a palm grove of Mollasani, North of Ahvaz, Iran, directions 31°34'58.6"N 48°53'15.3"E. The grove had not been sprayed. Population D (Ramin University) was collected from orange trees, the same location as population C, in late August 2017, 3 weeks after spraying. The live termites were collected by fine brush. The collected termites were kept at -20 °C until use.

Bioassay

The adult foraging workers of mentioned populations were used in the experiments of this study. The termites were collected from the same colony of each population. Five concentrations of chlorpyrifos (40.8EC Shimi Keshavarz Company, Iran) including 500, 200, 80, 30, and 6 ppm for populations A and B, as well as 21, 8.1, 2.6, 0.9, and 0.3 ppm for populations C and D (with having mortality range between 10 and 90%), were prepared by serial dilution in distilled water. The concentrations were selected according to the preliminary test. One ml of each concentration and water (control) was dropped on the filter paper (Whatman No. 1) and placed in a clean 9-cm-diameter Petri dish. The papers were dried for one hour before the test. Each Petri dish (replicate) contained 8-12 treated workers that were immobilized by placing them in a frozen Icepack for the 30 s and there were six replicates for each concentration. Then the Petri dishes were placed in an incubator at 25 ± 2 °C and 70 ± 5% relative humidity. The mortality of individuals was recorded after 24 hours. Workers were probed with a fine brush to determine whether they were alive or dead.

Mortalities were corrected using Abbott's correction method (Abbott, 1925). SAS PROC PROBIT was used to determine the lethal concentration 50 (LC_{50}) based on the corrected mortalities.

Protein Assay

Total soluble protein content was determined according to the method of Bradford, (Bradford 1976) using bovine serum albumin as standard.

Glutathione S-Transferase Assay

The activity of GST was measured according to Habig et al. (1974). Briefly, 0.620 g of *M. diversus* was homogenized using 2500 µl ice-cold potassium phosphate buffer (0.1 M, pH 7.5). Then the homogenates were centrifuged at 13000 *g* for 15 minutes at 4°C and obtained supernatants were used for enzyme activity measurement. The supernatants were transferred to a freezer at -80 °C until activity measurements (Wellington, 2015).

Reduced glutathione (GSH), at a concentration of 10 mM, was dissolved in 2.5 ml phosphate buffer (0.1 M, pH 7.5 mM). The substrate 1-Chloro-2 and 4-dinitrobenzene (CDNB) was used according to the method described by Habig et al. 1974 with some modifications. In this study, CDNB (at a range of concentrations from 0.05 to 1.4 mM) was dissolved in 10 ml ethanol (96%).

Kinetic parameters of GST were determined as follows: 15 µl enzyme extract (or phosphate buffer as control), 135 µl phosphate buffer, 50 µl of CDNB with mentioned concentrations, and 100 µl of GSH (10 mM) were mixed, and the absorbance value at 340 nm wavelength was continuously measured (absorbance changes per minute) in a light spectrophotometer, Analytik Jena-SPEKOL 2000, for 5 minutes with 20 seconds intervals.

Acetylcholinesterase Assay

AChE activity measurement was performed based on a method described by Ellman et al. (1961). Briefly, 0.620 g of *M. diversus* was homogenized using 2500 µl of ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.1% Triton X-100, followed by centrifugation at 13000 *g* for 15 minutes at 4 °C. Obtained supernatants were transferred to a freezer at -80 °C for subsequent enzyme activity measurement. Acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), and propionylthiocholine iodide (PTC) substrates (at concentrations of 0.01 to 7.5 mM) were dissolved in 10 ml of distilled water. 0.02 g of DTNB and 0.0152 g of $NaHCO_3$ were dissolved in 10 ml of Tris-hydrochloride buffer (Serva Co.) and filtered.

To measure the kinetic parameters, 40 µl of enzyme extract (or phosphate buffer as control), 140 µl phosphate buffer (0.1 M, pH 7.5), and 20 µl DTNB were transferred to a cuvette, and then 40 µl substrate was added. The absorbance value was continuously measured at 340 nm wavelength in a light spectrophotometer for 2 minutes with 6 seconds intervals (Ellman et al., 1961).

Determination of Kinetic Parameters

The K_m and V_{max} values were determined by nonlinear regression of enzyme activity from Lineweaver–Burk plots using Lineweaver & Burk's (1934) method.

RESULTS

Toxicity Assay

LC_{50} values of chlorpyrifos for different populations of *M. diversus* are presented in Table 1. LC_{50} values in populations A and B were significantly more than those of

populations C and D, respectively. Based on LC_{50} values population C exhibited the lowest value indicating the highest susceptibility. The LC_{50} values of the insecticide in populations A, B, and D were 27.04, 21.85, and 1.29 folds of population C.

Protein Assay

Total protein contents for field populations A, B, C, and D are given in Table 2. Statistical analysis indicated a significant difference between the total protein contents of all four populations of termites. The protein content in populations A, B, C and D were 2.62, 2.38, 1.48, and 1.85 mg ml^{-1}, respectively. The highest protein content belonged to population A, approximately 1.77 times more than population C, with the lowest protein content.

Glutathione S-Transferase Activity

Investigation of changes in the substrate concentration of CDNB with GSTs activity at various concentrations (0.05-1.4 mM) showed a significant difference in all populations. The highest activity of GSTs was observed at 1 mM substrate in which the values were significantly different in the four populations (Fig. 1). Significant differences were observed between populations B and C, which seems to have the highest kinetic difference.

V_{max} and K_m values of GSTs were determined by the kinetic analysis of the four populations using CDNB substrate. The highest and lowest hydrolysis efficiencies (V_{max}) were 0.0578 and 0.0142(µmol/min/mg protein) for populations C and B, respectively. V_{max} and K_m values of all populations were significantly different. The highest and lowest K_m values were related to the populations C and D with values of 0.66 and 0.20 µM, respectively (Table 3).

Table 1. LC_{50} values (ppm) of chlorpyrifos on four populations (A, B, C, and D) of *Microcerotermes diversus* tested in this study

Populations	N	LC_{50} (ppm)	Confidence interval Lower	Upper	Slope	Chi square	P-value	RR*
Am Altamir(A)	360	110.87	83.39	147.7	1.79±0.22	65.78	< 0.001	27.04
Am Altamir (B)	360	89.59	65.73	121.17	1.63±0.2	64.64	< 0.001	20.38
Mollasani (C)	360	4.1	3.07	5.52	1.7±0.2	73.56	< 0.001	-
Ramin University (D)	360	5.26	3.85	7.35	1.57±0.19	64.91	< 0.001	1.28

***** Resistance ratio (RR)= (LC_{50} of determined population/LC_{50} of susceptible population (Population C)

Table 2. Protein concentration (mg ml^{-1}) in four field populations of *Microcerotermes diversus* tested in this study

termite strain	Am Altamir(A) Mean±SE	Am Altamir (B) Mean±SE	Mollasani (C) Mean±SE	Ramin University(D) Mean±SE
Protein Concentration	2.62 ± 0.1^{a}	2.38 ± 0.16^{b}	1.48 ± 0.11^{d}	1.85 ± 0.09^{c}

The reported numbers are based on the mean ± standard deviation obtained from three replications. Means followed by different letters are significantly different at P <0.05 (Duncan's test).

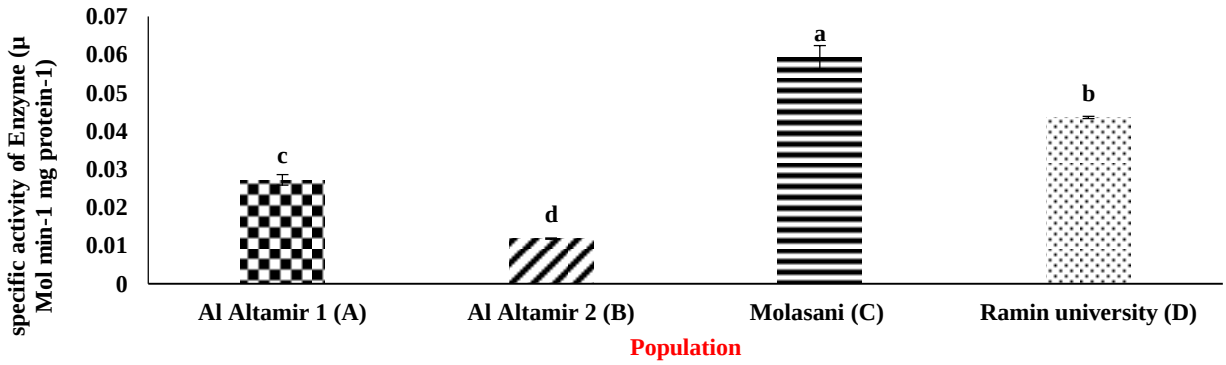

Fig. 1. The mean specific activity of glutathione-S-transferase at the concentration of 1 mM in four populations of *Microcerotermes diversus* tested in this study
The values of columns with different letters are significantly different at P <0.05 (ANOVA-Duncan post hoc-test).

Table 3. Kinetic parameters (mean ±SE) of glutathione-S-transferase in four populations of *Micrecerotermes diversus* tested in this study

Substrate	Property kinitic	termite strain			
		Am Altamir (A) Mean±SE	**Am Altamir (B)** Mean±SE	**Molasani (C)** Mean±SE	**Ramin University (D)** Mean±SE
CDNB	V_{max}	0.0301±0.001^{c}	0.0142±0.0007^{d}	0.0578±0.006^{a}	0.0429±0.005^{b}
	K_m	0.3407±0.028^{b}	0.4562±0.012^{b}	0.660±0.124^{a}	0.2094±0.054^{c}

V_{max}: Maximum speed value (micromolar per minute mg of protein).
K_m: Michaelis constant (micromolar). The reported numbers are based on the mean ± standard deviation obtained from three replications. Means followed by different letters are significantly different at P <0.05 (ANOVA-Duncan post hoc test).

Acetylcholinesterase Activity

The enzyme activity enhanced with increasing concentrations to 2.5 mM in all three substrates. But at concentrations above 2.5 mM, the incremental process was stopped. Thus, 2.5 mM was selected as an optimum concentration in which the highest activity of AChE was observed for all three substrates in different populations.

The specific activities of AChE at a concentration of 2.5 mM in ATC substrate were 0.108, 0.0476, 0.0134, and 0.0116 (µmol min^{1} mg protein^{-1}) in populations A, B, C, and D, respectively. The highest activity of AChE in ATC substrate was observed in population A, which was approximately 9.3 times more than that of population D, with the lowest activity (Fig. 2-A). Specific activities of AChE at a concentration of 2.5 mM in PTC substrate were 0.099133, 0.061233, 0.0105, and 0.012433 (µmol min^{-1} mg protein^{-1}) for populations A, B, C, and D, respectively. The highest enzyme activity of AChE in this substrate was observed in population A, which was approximately 7.97 times more than that of population D, with the lowest activity (Fig.2-B). The specific activity of the enzyme at a concentration of 2.5 mM in BTC substrate for populations A, B, C, and D were 0.0099333, 0.013933, 0.0071, and 0.0058333 (µmol min^{-1} mg protein^{-1}), respectively. The lowest enzymatic activity in BTC substrate was observed in population D and the highest in population A, approximately 1.7 times more than that of population D (Fig. 2-C)

V_{max} and K_m values of AChE for the termite populations with ATC, PTC, and BTC were determined using Lineweaver and Burk (1934) method. Maximum and minimum values of V_{max} belonging to populations A and D were 0.0151 and 0.0065 (µmol min^{-1} mg protein^{-1}), respectively. As the K_m value was highest for populations B and C and lowest for population A, it can be concluded that population A has had the highest affinity for ATC (Table 4).

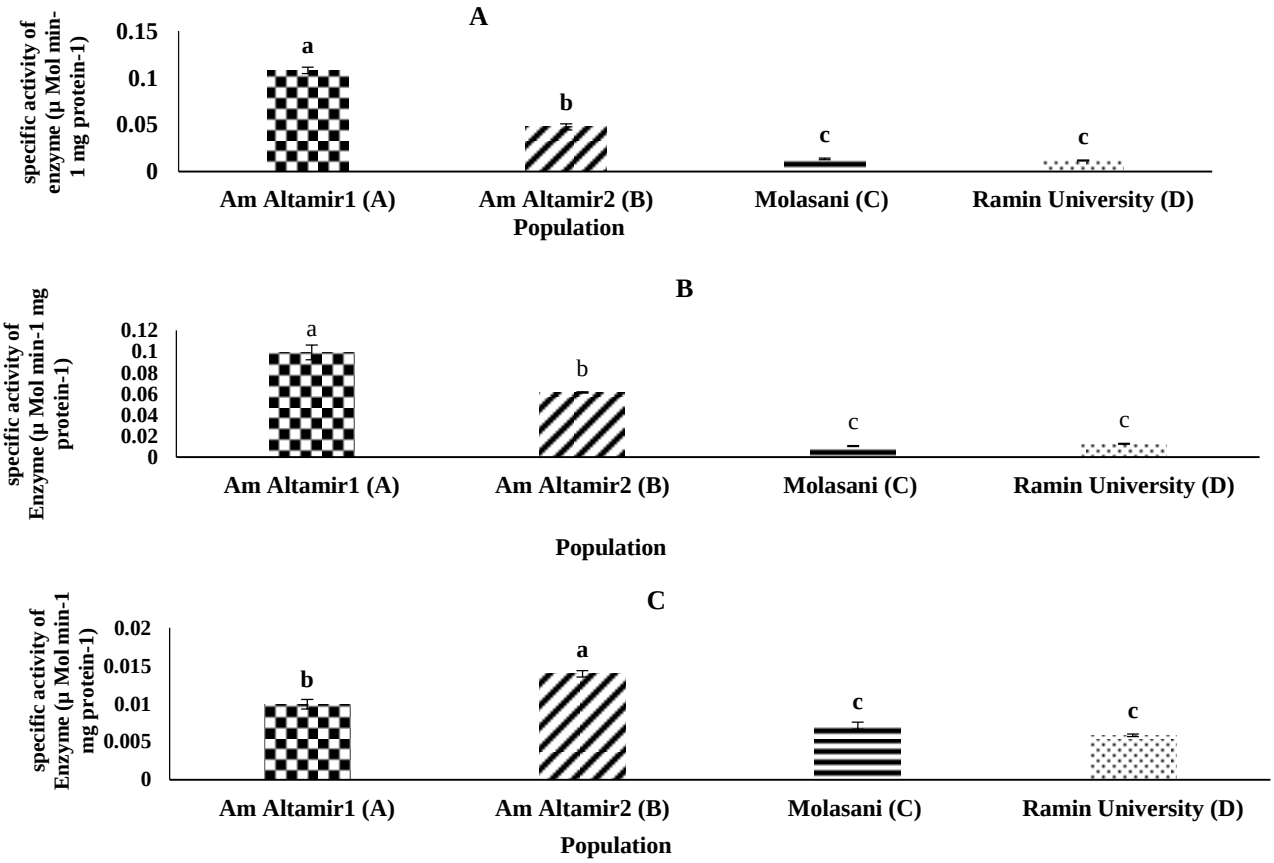

Fig. 2. The mean specific activity (±SE) of acetylcholinesterase at 2.5 mM concentration in A) acetylthiocholine iodide, B) propionylthiocholine iodide and, C) butyrylthiocholine iodide as substrates
The values of columns with the same letter are not significantly different (P <0.05, ANOVA-Duncan post hoc test).

Table 4. Kinetic parameters of acetylcholinesterase in four field populations of *Micrecerotermes diversus* tested in this study

Substrate	**Kinetic characteristic**	***Am Altamir (A)*** Mean±SE	***Am Altamir (B)*** Mean±SE	***Molasani* (C)** Mean±SE	**Ramin University (D)** Mean±SE
ATC	V_{max}	0.0551±0.0076[a]	0.0210±0.0005[b]	0.0112±0.0001[c]	0.0065±0.0004[c]
	K_m	0.2450±0.0323[a]	0.0835±0.0049[b]	0.0820±0.0039[b]	0.0408±0.0017[c]
BTC	V_{max}	0.0079±0.00081[b]	0.0117±0.00084[a]	0.0046±0.00065[c]	0.0047±0.00005[c]
	K_m	0.0814±0.0008[b]	0.0913±0.0101[ab]	0.0495±0.0052[c]	0.1022±0.0094[a]
PTC	V_{max}	0.0539±0.0053[a]	0.0264±0.0028[b]	0.0013±0.0092[c]	0.0101±0.0003[c]
	K_m	0.0740±0.010 [a]	0.0705±0.0058[a]	0.0317±0.0025[b]	0.0650±0.0063[a]

DISCUSSION

The results of the current study clearly demonstrated the higher LC_{50} values for susceptible populations compared to the other three populations. The resistance rate for different populations varied from 1.28 to 27.04. Similarly, Iqbal & Saeed (2013) showed that the LC_{50} values of *Microtermes mycophagus* D. (Isoptera: Termitidae) collected from different populations with various spraying histories to six insecticides are significantly different from susceptible populations.

Considering the protein nature of enzymes and the role and importance of various proteins in the body, protein assay is an important tool in most biochemical experiments (Robinson 2015). The results of this study showed that there were significant differences between the total protein contents of the termites of the four fields. Increased resistance to chlorpyrifos, which was observed in all populations, may be attributed to increased total protein levels. Similarly, Sabry & Abdel-Aziz (2013) showed higher total protein content in resistant colonies of *Pectinophora gossypiella* (Saunders) populations than in susceptible colonies. The results of this study are also in line with Khan Mirza et al. (2020) findings, which showed that the total protein content in the resistant population of *Aphis fabae* Scopoli was about 2.89-fold greater than that of the susceptible population and concluded that this observation may be attributed to gene amplification.

The AchE-specific activity was increased upon concentration enhancement of all three substrates (ATC, PTC, and BTC). This increasing trend continued up to 2.5 mM, but in concentrations above 2.5 mM, the process was stopped.

A significant difference in AchE-specific activity was observed between populations A and B (exposed to OP pesticides for a long period) and C and D (exposed to the same pesticides for a small period). From biochemical studies, it may be concluded that AChE has probably become insensitive in populations A and B. However, inhibition tests are required to prove the hypothesis. AChE is the primary and main enzyme responsible for the hydrolytic metabolism of the neurotransmitter acetylcholine and OP insecticides inhibit it (Robinson, 2015).

It has been reported that inhibition of enzyme activity at high substrate concentrations is an important indicator of AChE (Robinson, 2015). Previous studies have shown that the AChE of insects may inhibit in the presence of higher concentrations of its substrate in comparison with the AChE of vertebrates (Radic et al., 1991). It may be due to the apparent difference in the amino acid composition of the peripheral anions of vertebrates and insects (Radic et al., 1991). The results reported by Marcel et al. (1998), Yerushalmi & Cohen (2002), Zhu & Clark (1994) as well as Khan Mirza et al. (2020), who studied *Dorsaphila melanogaster* Meigen, *Aonidiella aurantii* Maskell, *Leptinotarsa desemlineata* Say, and *A. fabae*, respectively, indicate the importance of AChE in developing insecticide resistance to OP. The increased AChE-specific activity in resistant populations has also been observed in some other studies for the two-spotted spider mite (*Tetranychus urticae*), and *Liriomyza sativae* Blanchard (Zamani et al., 2014; Askari-Saryazdi et al., 2015).

Measurement of the kinetic parameters of enzymes also provides crucial information on the mechanisms of enzyme catalysis and their interactions with substrates, inhibitors, and pesticides (Robinson, 2015). V_{max} and K_m values of AChE for the termite populations with ATC, PTC, and BTC substrates showed a significant difference. Since the highest values of V_{max} and K_m were related to population Am Altamir (A), it can be concluded that among the studied populations, population A, with a higher resistance level, has a low affinity to the substrate. In accordance with this finding, higher values of K_m in resistant populations of insects and mites were also reported in studies reported by Vontas et al. (2001), Ghadamyari et al. (2008), and Zamani et al. (2014). The results of this study agree with Ghadamyari et al. (2008),

who showed that V_{max} values of resistant populations of in green peach aphids (Hemiptera: Aphididae) were more than those of susceptible populations with ATC and BTC substrates suggesting that the resistant population leads to a low enzyme affinity for the substrate tested.

The GSTs-specific activity with CDNB substrate had an increasing trend to 1 mM concentration, and the highest activity was observed in this concentration but enzyme activity stopped at 1 mM. A significant difference was observed between populations B and C which seems to have the highest kinetic difference. Similar results were found by Khan Mirza et al. (2020) who indicated that 1 mM was the optimum concentration to evaluate the activity of GSTs in *A. fabae*.

The results of the current study showed that GSTs specific activity didn't increase in populations A and B of the termites, which might be attributed to the diet type and applied pesticide. Usually, the OP pesticides cause significant changes in the kinetic parameters of AChE. On the contrary, it has been reported that the activity of GSTs in the *Aedes aegypti* (Diptera: Culicidae) population in dichlorvos application treatment was greater than that in the control population indicating GSTs can have a role in the detoxification of OP pesticides (Muthusamy et al., 2014). The difference observed in activity level of enzymes seems to be due to different pest species.

CONCLUSIONS

In conclusion, high resistance levels to chlorpyrifos were observed in some populations of *M. diversus*. The resistance in populations with a long history of spraying was significantly more than others. LC_{50} value in the resistant population A was 27.04 folds that of the susceptible population. It seems that the resistance of the termite to chlorpyrifos is related to the sensitivity of the AChE. Synergist's test data is required to find out the role of GSTs in the observed resistance.

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مقاله علمی- پژوهشی

مطالعه مقاومت به کلرپایریفوس در موریانه *Microcerotermes diversus* (Isoptera: Termitidae)

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واژه‌های کلیدی:
ارگانوفسفره‌ها
استیل کولین استراز
گلوتاتیون-اس-ترانسفراز
مقاومت به حشره‌کش‌ها
موریانه

چکیده - موریانه *Microcerotermes diversus* Silvestri موریانه‌ای مهم در استان خوزستان می‌باشد که دامنه‌ی جستجوگری وسیعی دارد. در سال‌های اخیر، مبارزه با موریانه‌ی زیرزمینی بیشتر بر مبنای کاربرد سموم شیمیایی بوده، که منجر به افزایش مقاومت به حشره‌کش‌ها در این آفت شده است. در این مطالعه، مقاومت جمعیت‌های مختلف این موریانه به حشره‌کش کلرپیریفوس با کمک تعیین مقادیر LC_{50} این حشره‌کش برای جمعیت‌های مختلف مورد آزمایش و محاسبه فعالیت دو آنزیم استیل کولین استراز و گلوتاتیون-اس- ترانسفراز مورد ارزیابی قرار گرفت. چهار جمعیت از *M. diversus* با تاریخچه متفاوتی از کاربرد حشره‌کش‌های فسفره آلی مورد استفاده قرار گرفتند. جمعیت A (ام‌اتمیر ۱)، جمعیت B (ام‌التمیر ۲) و جمعیت c (ملاثانی) از نخلستان‌هایی که به ترتیب دارای سابقه ۳۰ ساله و ۱۰ ساله سمپاشی با حشره کشهای فسفره آلی و بدون کاربرد این حشره کشها بودند جمع‌آوری شدند. جمعیت D (دانشگاه رامین) از درختان نارنج سه هفته بعد از سم‌پاشی با کلرپیریفوس جمع‌آوری شد. نتایج سنجش زیستی نشان داد که بیشترین و کمترین مقادیر LC_{50} به ترتیب در جمعیت‌های A و C وجود دارد. علاوه‌براین، میزان فعالیت استیل کولین استراز تعیین شده با استفاده از سوبستراهای استیل تیوکولین آیوداید، پروپینیل تیوکولین آیوداید و بوتریل‌تیونیل آیوداید در جمعیت‌های با سابقه کاربرد حشره‌کش کلرپایریفوس بیشتر بود (بیشترین میزان در جمعیت A). همچنین نشان داده شد که فعالیت استیل کولین استراز در جمعیت‌های با سابقه ۳۰ و ۱۰ ساله کاربرد حشره‌کش با سوبسترای استیل تیوکولین آیوداید بیشتر از غعالیت این آنزیم با سوبستراهای پروپینیل تیوکولین آیوداید و بوتریل‌تیونیل آیوداید بود. مقدار ثابت میکائلیس (K_m) و مقدار حداکثر سرعت (V_{max}) آنزیم استیل کولین استراز به عنوان دو عامل مهم کنتیک، بیانگر بیشترین تمایل این آنزیم به سوبسترا در جمعیت A بود.