

## Biochemical Resistance Mechanisms to Dimethoate in Cabbage Aphid *Brevicoryne brassicae* (L.) (Hom.: Aphididae)

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### ABSTRACT

Cabbage aphid, *Brevicoryne brassicae* (L.) (Hom.: Aphididae) is an important pest of crucifers and is controlled by different insecticides, especially dimethoate. The toxicity of dimethoate in six populations of the pest from different parts of Iran was assayed using Leaf-dip method. The bioassay results indicated significant difference in susceptibility to dimethoate among the six populations that were investigated. The highest level of resistance to dimethoate was obtained for Mehrshahr (Meh) population (RR= 91.25). Diethyl maleate (DEM), piperonylbutoxide (PBO), and triphenyl phosphate (TPP) suppressed the level of resistance to dimethoate, indicating the resistance to this insecticide was caused by glutathione S-transferases (GSTs), mixed function oxidases, and esterases, respectively. Cytochrome P450 monooxygenases and GSTs activity increased, respectively, 2.7 and 9.6-fold in resistant population compared with the susceptible one. When  $\alpha$ -naphthyl acetate was used as substrate, up to 4-fold increase in esterase activity was observed in resistant population. Moreover, 6.2-fold elevation in esterase activity was shown in resistant strain when  $\beta$ -naphthyl acetate was the substrate. Overall, the mechanisms of insecticide resistance in cabbage aphid populations from six regions of Iran were related to GSTs, esterase, and cytochrome P450 monooxygenases activities.

**Keywords:** Esterases, Glutathione S-transferases, Cytochrome P450 monooxygenases, Synergism

### INTRODUCTION

Cabbage aphid *Brevicoryne brassicae* Linnaeus (Hom.: Aphididae) is one of the most important pests that attack cruciferous crops. This aphid causes significant yield losses, lower quality, and even the complete loss of the plant (Ellis *et al.*, 1995).

Frequent applications of pesticides has caused a broad-spectrum resistance to them. It has been reported that *B. brassicae* has developed resistance to many insecticides including neonicotinoids (imidacloprid, thiamethoxam), pyrethroids (cypermethrin,

deltamethrin, bifenthrin) and organophosphates (profenofos, chlorpyrifos) (Ahmad and Akhtar, 2013). Approximately 70% of the pesticides in current use belong to organophosphate (Zamani *et al.*, 2014).

Pest resistance occurs by various mechanisms. The metabolic resistance is one of the most important mechanisms used by a group of enzymes including glutathione S-transferase (GSTs), esterases, and monooxygenase (Li *et al.*, 2007). P450s are a huge class of enzymes with many functions including biosynthesis and the metabolism of xenobiotics. Insect genomes contain from 46 to more than 150 P450

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genes that encode different P450 enzyme (Feyereisen, 2006). Microarray analysis in *Myzus persicae*, showed over-expression (22-fold) of a single P450 gene (CYP6CY3) that is associated with resistance to Neonicotinoid insecticides (Puinean et al. 2010). Glutathione S-transferase (GST) enzymes, which can be found in all eukaryotic organisms, work by conjugating xeno-biotics to reduced glutathione, therefore targeting them for more rapid degradation (Li et al., 2007). GSTs have been related with resistance to insecticides in insects (Enayati et al., 2005; Low et al., 2007). After ingestion of isothiocyanates, GST enzyme activity in *Myzus persicae* increased (Francis et al., 2005) which means GSTs are related to detoxification (Francis et al., 2001). Biochemical assays confirmed that the activities of glutathione S-transferases (GSTs), cytochrome P450 monooxygenase, and carboxylesterases (CarE) increased in *Aphis gossypii* resistant to dimethoate (Lokeshwari, et al., 2016). Many studies showed that resistance to organophosphates, carbamate, and pyrethroids was correlated with increasing nonspecific esterase activity (Abd El-Latif and Subrahmanyam, 2010). Increasing the activity of esterase has been reported in *Aphis pomi* resistant population to dimethoate (Tamaš et al., 2015).

In this study, we aimed to evaluate the susceptibility of six populations of *B. brassicae* to dimethoate and determine detoxification enzyme activities such as esterases, GSTs and cytochrome P450 monooxygenases in *B. brassicae* populations. Another objective was to study the effects of synergists for testing possible resistance mechanisms.

## MATERIALS AND METHODS

### Insects

Six populations of *B. brassicae* were obtained from cauliflower (*Brassica oleracea*) farm located in Falavarjan (32°33'19"N 51°30'35"E), Dezful

(32°22'57"N 48°24'07"E), Mehrshahr (35°48'03"N 50°54'19"E), Varamin (35°19'27"N 51°38'45"E), Kordan (35°55'53"N 50°49'52"E), and Tehran (Tarbiat Modares University farm, 35°41'21"N 51°23'20"E), Iran in 2016. All the populations, except Tehran population, were exposed to dimethoate before.

*B. brassicae* was reared on *Brassica oleracea* in a growth chamber with 50-70% relative humidity, 22±1 °C and 16/8 h (L:D) photoperiod (Kuśnierczyk et al., 2011). Apterous adult insect was used in all experiments.

## Bioassay

### Leaf-Dip Bioassay

In order to determine LC<sub>50</sub>, cauliflower, *B. oleracea* leaf disks (5 cm diameter) were dipped in five different concentrations of dimethoate (Sigma-Aldrich, USA) solutions containing 0.02% Tween-20 for 10 seconds. Distilled water containing 0.02% Tween-20 was used as control. After drying leaf disks at room temperature (RT), 80 apterous adult aphids were placed on treated leaves. Petri dishes containing leaves were kept in the growth chamber described above. Aphids with similar age were transferred to new growth chamber in order to rear them as distinct cohorts. After 24 hours, mortality of aphid was measured and the aphids that were not able to move by stimulating by camel hair brush were considered as dead. Experiment was repeated four times.

### Determination of Synergist Effects

Preliminary bioassay with different concentrations was done to choose appropriate dose of synergists. The highest dose of synergists [5 mg/L for piperonyl Butoxide (PBO) and triphenyl phosphate (TPP) and 10 mg/L for diethyl maleate (DEM)] in which the mortality was comparable with the control was used in the

bioassay. Each synergist was added to LC<sub>50</sub> concentration of dimethoate. Leaf-dip method was used for determination of the effect of dimethoate+ synergist. The synergists were added to LC<sub>50</sub> concentration of each population (the susceptible and the most resistant one). The mortality was recorded 24 h later.

### Esterase Activity

Esterase activity was determined using the method of Van Asperen (1962) with modification. Apterous adult aphids were used in this experiment. Aphids of similar age were transferred regularly to new growth chamber in order to rear them as distinct cohorts.

Fifty adults of each population were homogenized in 300  $\mu$ L of sodium phosphate buffer (10 mM, pH=7), containing 0.1% of Triton X-100 followed by centrifugation at 15000 g for 10 min at 4 °C. The supernatants were used for measuring the esterase activity.  $\alpha$ -Naphthyl acetate ( $\alpha$ -NA) and  $\beta$ -Naphthyl acetate ( $\beta$ -NA) were used as substrate. Then, 50  $\mu$ L fast blue RR (0.5 mg mL<sup>-1</sup> in buffer) was added. Finally, the released naphthol was measured continuously for 25 min at 450 nm using microplate reader (Bio Tek, USA). Four replicates were performed for each population.

Protein concentration was measured using Lowry *et al.* (1951) method.

### Glutathione S-Transferase Activity (GST)

GST activity was assayed as described by Habig *et al.* (1974). Fifty aphids were homogenized in 200  $\mu$ L of phosphate buffer (10 mM, pH=7) and homogenates centrifuged (10,000 g for 10 minutes at 4 °C). Ten  $\mu$ L of supernatants were transferred to new tubes containing two hundred microliter reaction mixtures [1mM of 1-chloro-2,4-dinitrobenzene (CDNB) and 5

mM of GSH solved in sodium phosphate buffer (0.1 M, pH=7)] were added. The absorbance changes were recorded continuously for 6 min at 340 nm, using microplate reader (Bio Tek, USA). Each population had four replication.

### Cytochrome P450 Activity

Cytochrome P450 monooxygenase activity assay was conducted according to De Sousa *et al.* (1995). Fifty aphids were homogenized at 4 °C in 1 mL of 0.1 M potassium /sodium phosphate [pH 7.2, containing 200 mM sucrose, 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA)] followed by centrifugation (5000g, 5 min, 4 °C).The supernatant was centrifuged (15,000g, 15 min) followed by another centrifugation (100000g , 60 min). Then, the pellet was solved in the phosphate buffer and was used as the enzyme source.

One hundred  $\mu$ L of the above mixture and 50  $\mu$ L of 0.1 M sodium/potassium phosphate buffer (pH 7.2) containing 1 mM NADPH and 0.4 mM 7-ethoxycumarin were added per well. The plate was shaken for 30 min at 30 °C and incubated with 0.5 units of glutathione reductase and 1.5 mM oxidized glutathione for 10 min at room temperature (RT). Finally, to stop the reaction, 150  $\mu$ L of acetonitrile (50%) in Trizma-base buffer (0.05 M, pH=10) was added. The released 7-ethoxycumarin was measured at 465 nm while exciting at 390 nm. For each population, four replicates were performed.

Protein concentration was determined using Bradford (1976) method.

### Data Analysis

Data analysis was done by using ANOVA and means were compared by Tukey's test ( $p < 0.05$ ) using SPSS (2004). LC<sub>50</sub> was determined using probit analysis using PoloPlus computer software (LeOrá Software 2007).



## RESULTS

### Dimethoate Resistance Levels

The LC<sub>50</sub> values of dimethoate for the six populations of *B. brassicae* obtained from different regions of Iran are presented in Table 1. The populations clearly showed significant differences in LC<sub>50</sub>. Comparison among the populations revealed that the highest and lowest LC<sub>50</sub> belonged to Tehran (Teh) population (4 mg/L) and Mehrshahr (Meh) population (365 mg/L), respectively. In other words, the resistance level for Meh was 91.25-fold higher than that of Teh population. Therefore, Teh and Meh populations were considered as susceptible and resistant populations, respectively. Varamin (Var), Falavarjan (Fal), Kordan (Kor) and Dezful (Dez) populations showed 50, 25, 12.5, 2.5-fold resistance ratio in comparison with Teh as susceptible population, respectively (Table 1).

### Synergists

Synergistic effect of PBO, DEM and TPP were measured in the susceptible (Teh) and resistant (Meh) populations, to find the involvement of cytochrome P450

monooxygenase, GSTs and esterase in the resistance mechanism to dimethoate in *B. brassicae*, respectively. The LC<sub>50</sub> of dimethoate decreased from 365 mg/L in dimethoate-treated in Meh population to 64 mg/L in dimethoate-DEM treated population. Moreover, PBO and TPP had a significant effect on LC<sub>50</sub> in Meh as a resistant population, while in Teh (susceptible) population, there was no significant difference between dimethoate-treated and dimethoate+synergist treated populations. On the other hand, DEM, TPP and PBO did not have any synergistic effect in the susceptible population (Table 2).

### Esterase Activity

The activity of esterase was measured using  $\alpha$ -NA and  $\beta$ -Na, as substrate. When  $\alpha$ -NA was used as substrate, the highest esterase activity among the populations was observed in Meh populations, which were almost 4-fold higher than in the susceptible population (Teh) (Figure 1A). Results indicated that when the substrate was  $\beta$ -NA, Meh had higher esterase activity (6.2-fold) compared with the susceptible population (Teh) (Figure 1B).

### GST Activity

Among the populations, the highest GST activity was observed in Meh, which was

**Table 1.** Susceptibility of six populations of *Brevicoryne brassicae* to dimethoate using leaf-dip method.

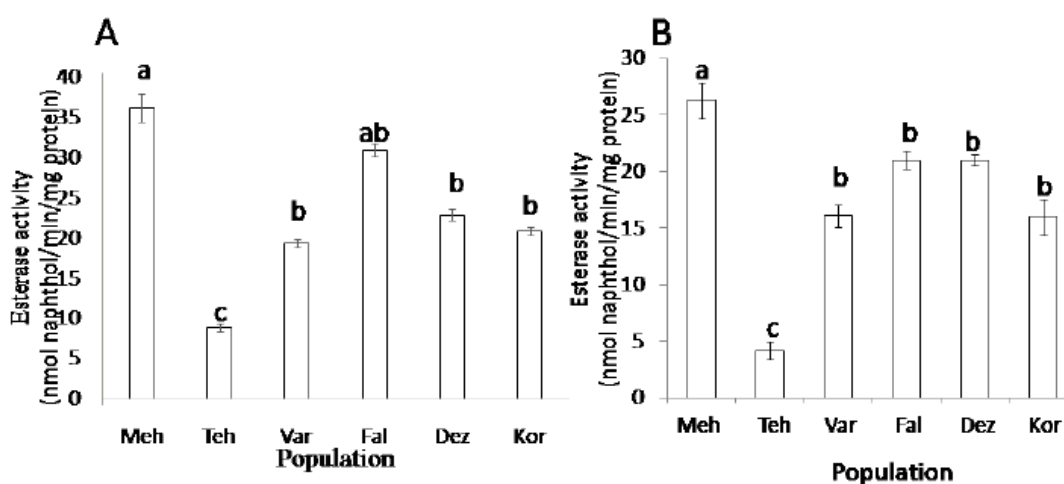
Population <sup>a</sup>	n <sup>b</sup>	LC <sub>50</sub> (LCL-UCL) <sup>c</sup> (mg/l)	slope±SE	$\chi^2$ (df)	RR <sup>d</sup>
Meh	320	365(295-425)	0.580±0.043	2.4(3)	91.25 <sup>a</sup>
Var	320	200(195-295)	0.313±0.016	1.9(3)	50 <sup>b</sup>
Fal	320	100(65-119)	0.244±0.092	1.3(3)	25 <sup>b</sup>
Kor	320	50(32-108)	0.174±0.022	0.7(3)	12.5 <sup>c</sup>
Dez	320	10(7-17)	0.200±0.049	1.3(3)	2.5 <sup>c</sup>
The	320	4(2-6)	0.165±0.012	1.3(3)	... <sup>d</sup>

<sup>a</sup> Meh: Mehrshahr; Va: Varamin; Fal: Falavarjan; Kor: Kordan; Dez: Dezful; Teh: Tehran; <sup>b</sup> Number of insect <sup>c</sup> LCL: lower confidence limit at 95%; UCL: upper confidence limit at 95%; <sup>d</sup> Resistance Ratio: LC<sub>50</sub> of resistant population / LC<sub>50</sub> of susceptible population. Means with same letter are not significantly different at p< 0.05.

**Table 2.** Effect of PBO, DEM and TPP on dimethoate resistant and susceptible populations of *Brevicorye brassicae*.

Population <sup>a</sup>	synergist	n <sup>b</sup> LC <sub>50</sub>	(LCL-UCL) <sup>c</sup> (mg/l)	slope±SE	$\chi^2$ (df)	RR <sup>d</sup>	SR <sup>e</sup>
Meh		150	365(295-425)	0.580±0.043	2.4(3)	91.25	.....
	DEM	150	64 (47-81)	0.147±0.053	0.49(3)	16	5.70
	PBO	150	120 (103-137)	0.294±0.098	0.79(3)	30	3.04
	TPP	150	110 (84-142)	0.202±0.067	0.70(3)	27.5	3.31
Teh		150	4(2-6)	0.165±0.012	1.3(3)	.....	.....
	DEM	150	2(1-3)	0.83±0.011	0.65(3)	.....	2
	PBO	150	4.3(3.3-5.3)	0.163±0.022	1.40(3)	.....	0.93
	TPP	150	1.75(0.7-2.8)	0.79±0.010	0.57(3)	.....	2.28

<sup>a</sup> Meh: Mehrshahr; Teh: Tehran <sup>b</sup> Number of insect <sup>c</sup> LCL: lower confidence limit at 95%; UCL: upper confidence limit at 95% <sup>d</sup> Resistance Ratio: LC<sub>50</sub> of resistant population / LC<sub>50</sub> of susceptible population. <sup>e</sup> Synergist Ratio: LC<sub>50</sub> of dimethoate alone / LC<sub>50</sub> of dimethoate+synergists. Means with same letter are not significantly different at p< 0.05.



**Figure 1.** Esterase activity of adult *Brevicorye brassicae* of six populations using  $\alpha$ -NA (A) and  $\beta$ -NA (B) as substrate. The means with different letters are significantly different at p < 0.05. Meh: Mehrshahr; Va: Varamin; Fal: Falavarjan; Kor: Kordan; Dez: Dezfool; Teh: Tehran.

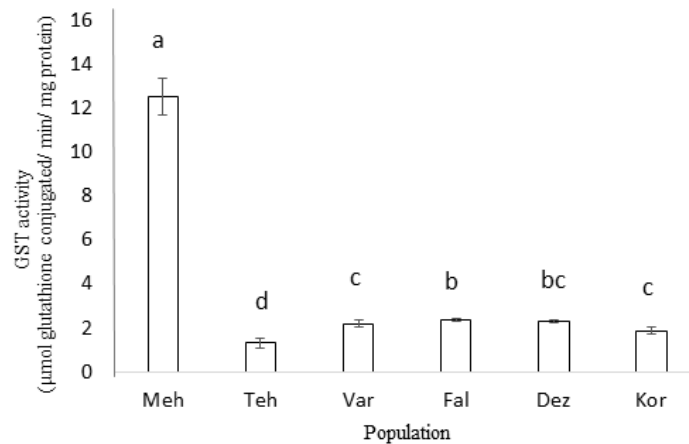
about 9.6-fold higher than the susceptible population (Teh). There was no significant difference among Kor, Dez and Var populations in GST activity. Moreover, there was no significant difference between Fal and Dez population (Figure 2).

#### Cytochrome P450 Monooxygenase Activity

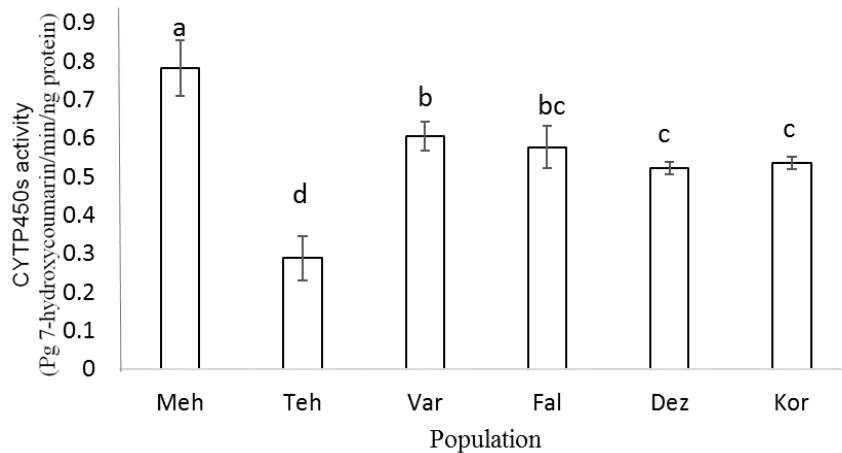
Analysis of cytochrome P450 monooxygenase activity of the six *B. brassicae* populations confirmed that there

were significant differences among the populations. The Meh population showed the highest level of enzyme activity, which was approximately 2.7-fold higher than that of the Teh (susceptible) population. There was no significant difference among Kor, Dez and Fal populations in cytochrome P450 monooxygenase activity. Moreover, there was no significant difference between Fal and Var population (Figure 3).

#### DISCUSSION



**Figure 2.** GSTs activity of of adult *Brevicoryne brassicae* of sixpopulations. The means with different letters are significantly different at  $p < 0.05$ . Meh: Mehrshahr; Va: Varamin; Fal: Falavarjan; Kor: Kordan; Dez: Dezfool; Teh: Tehran.



**Figure 3.** Cytochrome P450 monooxygenase activity of adult *Brevicoryne brassicae* of six populations. The means with different letters are significantly different at  $p < 0.05$ . Meh: Mehrshahr; Va: Varamin; Fal: Falavarjan; Kor: Kordan; Dez: Dezfool; Teh: Tehran.

Organophosphates are pest-control agents heavily used all over the world (Burke *et al.*, 2017). Among the six populations, Meh showed the highest level of resistance to dimethoate. In other words, the resistance level for Meh was 91.25-fold higher than that of susceptible population (Teh). Repetitive application of dimethoate in Mehrshahr for controlling aphids may cause the highest level of resistance in this population (Tabashnik *et al.*, 2014). No insecticide was used for controlling pests in Teh, the susceptible population, for several years.

Insecticide resistance has a variety of mechanisms, such as enhancement of metabolic detoxification, accelerated excretion of insecticides, reduction of penetration rate, and target site insensitivity (Pang *et al.*, 2016). Esterase, GSTs and cytochrome P450 monooxygenase are the most important mechanisms for insect resistance because of their significant role in detoxifying insecticides (Ghadamyari *et al.*, 2008). Esterases are a large family of enzymes that hydrolyze amide, carboxylester and thioester bonds in many compounds. They are more important in

insects because of their role in the detoxification of organophosphates (Mukanganyama *et al.*, 2003). Here, detoxifying mechanisms of dimethoate resistance were investigated. This mechanism was investigated *in vivo* by studying the effect of synergists on resistant and susceptible population. Dimethoate toxicity increased 3.31 fold in the presence of TPP. Moreover, compared with the susceptible population, esterase activities in the resistant population were approximately 4 and 3-fold, when  $\alpha$ -NA and  $\beta$ -NA were used as substrate, respectively. Therefore, our result confirmed that esterase participated in resistance of *B. brassicae* to dimethoate. Increasing the activity of esterase is a common mechanism of resistance against many groups of insecticides including organophosphate. Previously, increase in esterase activity was shown in *Aphis pomi* populations resistant to dimethoate (Tamaš *et al.*, 2015).

GSTs play an important role in the detoxification of almost all groups of insecticides (Alizadeh *et al.*, 2011), specially organophosphates (You *et al.*, 2015). GSTs have been associated in many cases of organophosphate resistance (Enayati *et al.*, 2005). The conjugation of glutathione to organophosphate insecticides caused detoxification via two pathways: O-dealkylation and O-dearylation. In the first pathway, glutathione conjugates with the alkyl portion of organophosphate (Oppenoorth *et al.*, 1979). In the second pathway, the glutathione reacts with the leaving group (Chiang and Sun, 1993). Lokeshwari *et al.* (2016) confirmed the increase in GSTs in *Aphis gossypii* resistant to dimethoate. Here, we also showed 9.6-fold increase in GSTs activity in resistant population (Meh) compared with the susceptible one (Teh). Moreover, dimethoate toxicity increased by 5.7-fold in the presence of DEM in Meh population.

The mixed function oxidases (MFO) are also mentioned to have an important role in detoxification of several insecticides. The oxidation of insecticides is catalyzed by the

cytochrome P450s genes, so, it is the only metabolic system that is involved in resistance to all groups of insecticides (Hemingway *et al.*, 2004). Cytochrome P450 monooxygenase introduces polar groups into the substrate and makes them suitable for conjugation at Phase II of detoxification (Karuppaiah *et al.*, 2017). Some previous studies showed the relationship between resistance to dimethoate and increasing of cytochrome P450 monooxygenase activity in resistant *Aphis gossypii* (Lokeshwari, *et al.*, 2016). Here, it was shown that cytochrome P450 monooxygenase activity in resistant population (Meh and Var) was up to 2.78-fold higher than that in Teh population as the susceptible one. Moreover, the toxicity of dimethoate increased by 3-fold in the presence of PBO. Therefore, this enzyme seems to be important in resistance to dimethoate.

The expression levels of cytochrome P450 monooxygenase, esterase, and GSTs genes will help us to understand the roles of these genes in dimethoate resistance in *B. brassicae*. Moreover, research on susceptibility of *B. brassicae* populations to other common insecticides to find out whether or not resistance to dimethoate conferred resistance to them as well will help us to manage this important pest.

In conclusion, the presence of elevated detoxification enzyme levels indicated the multiple resistance mechanism in resistant populations of *B. brassicae*. This may be an obstacle to the appropriate control of this pest.

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### ساز و کارهای بیوشیمیایی مقاومت به دیمتوات در شته کلم (*Brevicoryne brassicae* L.)

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#### چکیده

شته کلم از مهمترین آفات چلیپاییان بوده و توسط حشره کش های مختلفی از جمله دیمتوات کنترل می شوند. در ابتدا سمیت دیمتوات در شش جمعیت از این آفت از ۶ استان ایران به روش غوطه ور کردن برگ انجام شد. نتایج سنجش بیانگر حساسیت های مختلف نسبت به دیمتوات در این شش جمعیت بود به طوری که بالاترین نسبت مقاومت در جمعیت مهرشهر ( $RR=91.25$ ) به دست آمد. DEM، PBO و TPP میزان مقاومت به دیمتوات را کاهش دادند که به ترتیب نشانگر تاثیر گلوکاتیون-اس ترانسفراز (GSTs)، سیتوکروم پی ۴۵۰ مونواکسیژناز و استراز در مقاومت به دیمتوات



بود. فعالیت سیتوکروم پی ۴۵۰مونواکسیژناز و GSTs در جمعیت مقاوم به ترتیب دو و هفت دهم و شش و دو دهم برابر جمعیت حساس بود. فعالیت استراز در جمعیت مقاوم زمانی که از سوبسترای آلفا نفتیل استات استفاده شد، چهار برابر جمعیت حساس بود. زمانی که از سوبسترای بتا نفتیل استات استفاده شد فعالیت استراز شش و دو دهم برابر جمعیت حساس بود. در نتیجه می توان نتیجه گرفت که ساز و کار مقاومت در شته کلم نسبت به دیمتوات به دلیل افزایش فعالیت آنزیم های GSTs ، سیتوکروم پی ۴۵۰مونواکسیژناز و استراز می باشد.