

## Synergistic Effects of Amitraz on Imidacloprid and Malathion against Cotton Aphid, *Aphis gossypii* (Hem: Aphididae)

A. Shojaei<sup>1\*</sup>, K. Talebi Jahromi<sup>1</sup>, V. Hosseininaveh<sup>1</sup>, and G. Sabahi<sup>1</sup>

### ABSTRACT

For more than a century, chemical insecticides have been the most primary tool used by growers to control the cotton aphid, *Aphis gossypii*, as an important polyphagous pest worldwide. The application of insecticide mixtures through different modes of action is currently favored for resistance management of this pest. In this study, the synergistic interactions between amitraz with each of the two commonly used insecticides, i.e. imidacloprid and malathion, were studied using *A. gossypii* as target pest. The effects of amitraz combination on the activity of three detoxifying enzymes of cotton aphids were then evaluated using physiological assays. The synergistic effects of amitraz on imidacloprid were observed at all Lethal Concentrations (LC<sub>10</sub>-LC<sub>90</sub>), while, for malathion it was observed at concentrations higher than LC<sub>30</sub>. The highest synergist ratio in the mixture of amitraz with malathion (LC<sub>90</sub>) and imidacloprid (LC<sub>10</sub>) was 1.5 and 3.09, respectively. The inhibition of Glutathione S-Transferase (GST) activity seems to be the main reason for amitraz to impose its synergistic effects.

**Keywords:** Detoxifying enzymes, Insecticides, Lethal concentrations, Synergism.

### INTRODUCTION

Cotton aphid, *Aphis gossypii* Glover (Hem: Aphididae), is an important polyphagous pest of agriculture worldwide that attacks more than 300 plant species including cucurbits, tomato, and cotton under field and greenhouse conditions (Fuller *et al.*, 1999). In the United States, *A. gossypii* has been considered as one of the most important pests of cotton in term of yield loss in 2002 and 2003, respectively (Williams, 2003, 2004).

For more than a century, chemical insecticides have been the most primary tool used by growers to control it worldwide (Shi *et al.*, 2011). Since the late 1980's, however, it emerged as an important pest, while most

recommended insecticides failed to provide satisfactory control (Hardee and Ainsworth, 1993). Different populations are currently known to have developed different degrees of resistance against a wide variety of synthetic insecticides, including organophosphates, pyrethroids and neonicotinoids (Kerns and Gaylor, 1992; Amad *et al.*, 2003; Nauen and Elbert, 2003; Cao *et al.*, 2008). Its ability to develop rapid resistance to insecticides arises from its high reproductive rate and short life cycle (O'Brien and Graves, 1992).

At the biochemical level, metabolic resistance to insecticides typically involves an increases in the metabolic capabilities of detoxifying enzymes, such as Monooxygenases (MOF), esterases and

<sup>1</sup> Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, P. O. Box: 31587-77871, Karaj, Islamic Republic of Iran.

\*Corresponding author; e-mail: amin\_shojaei@ut.ac.ir



Glutathione S-Transferases (GSTs) as well as a decrease in sensitivity of the target site (Plapp, 1984; Li *et al.*, 2007). Accurate identification of enzymes involved in insecticide detoxification is therefore of special importance to develop new classes of pesticides for use in insecticide resistance management. The combination of two or more insecticides with different modes of action would be the best strategy to slow the rate of resistance development by insect pests (Cloyd *et al.*, 2007; Darriet and Chandre, 2013; Basit *et al.*, 2013).

In a recent field study, the use of a mixture of some commercial insecticides was an appropriate strategy to obtain satisfactory control as well as to mitigate resistance development by cotton aphids in Ethiopia (Shonga *et al.*, 2013). Apart from this, the application of insecticide mixtures may result in synergistic effects on target pest which increases control efficacy, while decreases cost and toxicity on non-target organisms. Synergism occurs when the combined effect of the mixture is stronger than the sum of the single effects (Corbel *et al.*, 2003; Corbel *et al.*, 2006).

Imidacloprid and malathion are among the most commonly used insecticides against aphids, including *A. gossypii*, in Iran (Anonymous, 2011). Like other neonicotinoids, imidacloprid is an insect neurotoxin, which acts selectively on insect nicotinic acetylcholine receptors with low toxicity to vertebrates (Nauen and Denholm, 2005). Malathion is an organophosphate of relatively low human toxicity which binds irreversibly to acetylcholinesterase (Tomlin, 2009). Amitraz is a non-systemic pesticide which acts on octopamine receptors of the central nervous system. It is often used in combination with other pesticides due to its synergistic interactions (Prullage *et al.*, 2011; Rodriguez *et al.*, 2013).

In this study, the efficacy of imidacloprid and malathion, either individually or in combination with amitraz, in the control of

cotton aphids was investigated under laboratory conditions. Moreover, some physiological assays were carried out to compare the activity of common detoxifying enzyme groups of survived aphids, either when they were treated singly or when treated in combination with amitraz.

## MATERIALS AND METHODS

### Plants and Insects

Adults of *A. gossypii*, were collected from a cucumber greenhouse in 2013, Karaj, Iran, and were transferred to the Toxicology Laboratory of the University of Tehran, Iran, where they were identified at species level and monitored for two days to remove any infection by parasitoids. A stock population was then established in a greenhouse under controlled conditions ( $25\pm 2^{\circ}\text{C}$ ,  $65\pm 5\%$  RH, and 16 L: 8 D h) with cucumber plants (*Cucumis sativus* cultivar Soltan) used as the host plants (Davoodi Dehkordi and Sahragard, 2013). Six-leaf stage plants were used for aphid rearing.

### Chemicals

Technical imidacloprid powder (98%), malathion (95%), and amitraz (98%), used in this study, were obtained from Giah company, Iran. Stock solutions were prepared by dissolving the technical grade pesticides in acetone.

### Bioassay

Cucumber leaves were broadened upside down on a 1.2% agar layer in 9 cm diameter Petri dishes with one central screened hole (3 cm diameter) on its lid for ventilation. Fifteen one-day old apterous adults were released on cucumber leaf discs. Topical

application method was used by means of a microapplicator (Burkard Ltd., England) for pesticide treatment (Ghadamyari *et al.*, 2008). Four replicates were considered for each bioassay. To prevent quick evaporation and convenient usage, the solutions were prepared in a mixture of acetone and distilled water (70:30 v/v). 0.25  $\mu\text{L}$  of each insecticide solution was placed on abdominal tergum of each aphid using microapplicator and mortality was recorded after 24 hours of exposure. A mixture of acetone and distilled water (70:30 v/v) was used to treat aphids as control.

### Synergistic Effects

In preliminary bioassays, the highest concentrations of amitraz (90  $\text{mg L}^{-1}$ ) which produced no mortality ( $LC_0$ ) on adult cotton aphids were determined. The aphids were then exposed to five different concentrations of imidacloprid and malathion individually (as positive control) and in combination with  $LC_0$  amitraz. A log-probit analysis was performed for each insecticide individually and in combination and their slopes were compared with a chi-squared parallelism test. Synergism Ratios (SR) were calculated in order to determine the magnitude of change in efficacy of each insecticide occurring in combination with amitraz. Synergistic Ratios (SR) were calculated using the following formula:

$$SR = \frac{LC_{\text{Insecticide without } LC_0 \text{ Amitraz}}}{LC_{\text{Insecticide with } LC_0 \text{ Amitraz}}}$$

Where,  $SR > 1$  and  $SR < 1$  show synergistic and antagonistic interactions, respectively (Corbel *et al.*, 2003). Detoxifying Enzyme Assay The activity of the three main detoxifying enzyme groups, Glutathione S-Transferases (GSTs), esterases, and general oxidases were quantified in the cotton aphids treated by  $LC_{50}$  of imidacloprid and malathion, in combination with amitraz and individually. Ten aphids, which survived at mentioned lethal concentrations, were

homogenized in phosphate buffer 0.1M (180  $\mu\text{L}$ ) at 4°C. The homogenate was centrifuged using a microcentrifuge (Eppendorf 5417 R) at 10,000 $\times g$  for 10 minutes at 4°C. The supernatant was used as enzyme source.

Glutathione S-transferase activity was measured according to the method of Habig *et al.* (1974). Briefly, enzyme samples (15  $\mu\text{L}$ ) were placed in microplate wells containing 200  $\mu\text{L}$  Chloro-DiNitroBenzene solution (CDNB; 63 mM solved in methanol) and reduced Glutathione (GSH; 10 mM) with Ratio of 50:1. Finally, the absorbance was read at 340 nm every 30 seconds for 5 minutes (Habig *et al.*, 1974).

Esterase activity was quantified following Van Asperen method (1962). Ten aphids were homogenized with 1% Triton X-100 in phosphate buffer (0.1 M, pH 7). Thirty  $\mu\text{L}$  of 1-naphthyl acetate and 2-naphthyl acetate (solved in acetone) were used as the substrate that was diluted by phosphate buffer 0.02M (ratio 1:99). The enzyme samples (15  $\mu\text{L}$  for 1-naphthyl and 10  $\mu\text{L}$  for 2-naphthyl) were then introduced to microplate wells containing 1-NA or 2-NA (200  $\mu\text{L}$ ) and 50  $\mu\text{L}$  fast blue RR (solved in distilled water ratio of 1:10). The absorbance was read at 450 nm for 1-naphthyl and 540 nm for 2-naphthyl every 2 minutes for 10 minutes (Van Asperen, 1962).

The amount of general oxidase was measured using Brogdon *et al.* (1997) method. Briefly, the enzyme samples (20  $\mu\text{L}$ ) were introduced into microplate wells containing 80  $\mu\text{L}$  potassium phosphate buffer (0.625 M, pH 7.2), 200  $\mu\text{L}$  of 3,3',5,5'-tetramethylbenzidine (solved in methanol), sodium acetate buffer and 25  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  (3%). After two hours of incubation in darkness at 25°C, the absorbance was read at 450 nm and its value was calculated by cytochrome C curve (Brogdon *et al.*, 1997). The total protein concentration of samples was measured according to Bradford (1976) method using Bovine Serum Albumin (BSA) as standard.



## Data Analysis

Data were analyzed using POLO-Plus 2.0 and SPSS 22.0 software. Analysis Of Variance (ANOVA) was conducted to compare the mean values of enzyme activity among different treatments ( $P \leq 0.05$ ).

respectively. The slope of the regression line for malathion was  $2.1 \pm 0.28$  ( $\chi^2 = 7.44$ ,  $df = 18$ ,  $P < 0.01$ ) which was lower than this value ( $2.72 \pm 0.48$ ) for malathion-amitraz mixture ( $X^2 = 12.85$ ,  $df = 18$ ,  $P < 0.01$ ).

## RESULTS

### Synergistic Interaction in Malathion-Amitraz Mixture

We found significant synergistic interactions between malathion and amitraz at all lethal concentrations higher than  $LC_{30}$  (Table 1). The synergism was positively correlated with the dose of malathion and the lowest and the highest synergisms were detected at  $LC_{40}$  and  $LC_{90}$  malathion,

### Synergistic Interaction in Imidacloprid-Amitraz Mixture

Amitraz imposed synergistic effects in combination with imidacloprid at all lethal concentrations (Table 2). In contrast to malathion, a negative correlation was found between the dose of exposed imidacloprid and the intensity of synergism, such that the lowest and the highest synergisms appeared at  $LC_{90}$  and  $LC_{10}$  mixture, respectively (Table 2). The slope of the regression line was  $2.19 \pm 0.32$  ( $X^2 = 9.35$ ,  $df = 18$ ,  $P < 0.01$ ) in imidacloprid treatment and  $1.6 \pm 0.26$  ( $X^2 = 19.39$ ,  $df = 18$ ,  $P < 0.01$ ) in imidacloprid-

**Table 1.** Effect of malathion without and with amitraz against *A. gossypii*.

Lethal Concentration	Malathion ( $\text{mg L}^{-1}$ )		Synergist ratio <sup>c</sup>
	Without amitraz (CI 95%)	With amitraz <sup>b</sup> (CI 95%)	
$LC_{10}$	38.54 (22.6-53.3)	48.54 (27.5-65.1)	0.79
$LC_{20}$	62.44 (42.8-79.6)	70.43 (47.9-87.6)	0.88
$LC_{30}$	88.41 (67.0-107.8)	92.12 (70.1-110.3)	0.95
$LC_{40}$	119.00 (96.1-142.5)	115.87 (94.5-138.0)	1.02
$LC_{50}$	157.11 (130.8-190.8)	143.57 (120.5-176.4)	1.09
$LC_{60}$	207.41 (172.2-263.8)	177.90 (148.4-233.6)	1.16
$LC_{70}$	279.18 (225.2-382.7)	223.76 (181.1-323.0)	1.24
$LC_{80}$	395.30 (302.8-602.4)	292.66 (225.1-479.2)	1.35 <sup>a</sup>
$LC_{90}$	640.32 (450.2-1146.0)	424.66 (300.6-838.1)	1.5 <sup>a</sup>

<sup>a</sup> Significantly different ( $P \leq 0.05$ ) between the mixture and pesticide without amitraz at the same lethal concentration. <sup>b</sup> Concentration of amitraz was  $90 \text{ mg L}^{-1}$ . <sup>c</sup> Synergistic ratio: Calculated by dividing the  $LC_n$  of malathion without amitraz by the  $LC_n$  of malathion with amitraz.

**Table 2.** Effect of imidacloprid without and with amitraz against *A. gossypii*.

Lethal concentration	Imidacloprid (mg L <sup>-1</sup> )		Synergist ratio <sup>c</sup>
	Without amitraz (CI 95%)	With amitraz <sup>b</sup> (CI 95%)	
<i>LC</i> <sub>10</sub>	200.65 (101.05-298.74)	64.76 (22.45-110.84)	3.09 <sup>a</sup>
<i>LC</i> <sub>20</sub>	318.23 (190.47-434.81)	121.80 (58.19-182.54)	2.612 <sup>a</sup>
<i>LC</i> <sub>30</sub>	443.79 (297.86-575.62)	192.08 (112.82-268.16)	2.31 <sup>a</sup>
<i>LC</i> <sub>40</sub>	589.65 (430.70-741.33)	283.46 (191.10-387.13)	2.08 <sup>a</sup>
<i>LC</i> <sub>50</sub>	769.03 (596.10-957.79)	407.82 (295.29-577.93)	1.88 <sup>a</sup>
<i>LC</i> <sub>60</sub>	1002.99 (802.20-1272.62)	586.74 (428.10-919.55)	1.70 <sup>a</sup>
<i>LC</i> <sub>70</sub>	1332.63 (1066.19-1783.12)	865.90 (606.14-1588.25)	1.53 <sup>a</sup>
<i>LC</i> <sub>80</sub>	1858.42 (1440.87-2731.62)	1365.44 (880.90-3112.25)	1.36 <sup>a</sup>
<i>LC</i> <sub>90</sub>	2947.47 (2124.72-5081.80)	2568.0 (1442.0-8116.2)	1.14 <sup>a</sup>

<sup>a</sup> Significantly different ( $P \leq 0.05$ ) between the mixture and pesticide without amitraz at the same lethal concentration. <sup>b</sup> Concentration of amitraz was 90 mg L<sup>-1</sup>. <sup>c</sup> Synergistic ratio: Calculated by dividing the *LC*<sub>*n*</sub> of malathion without amitraz by the *LC*<sub>*n*</sub> of malathion with amitraz.

amitraz mixture.

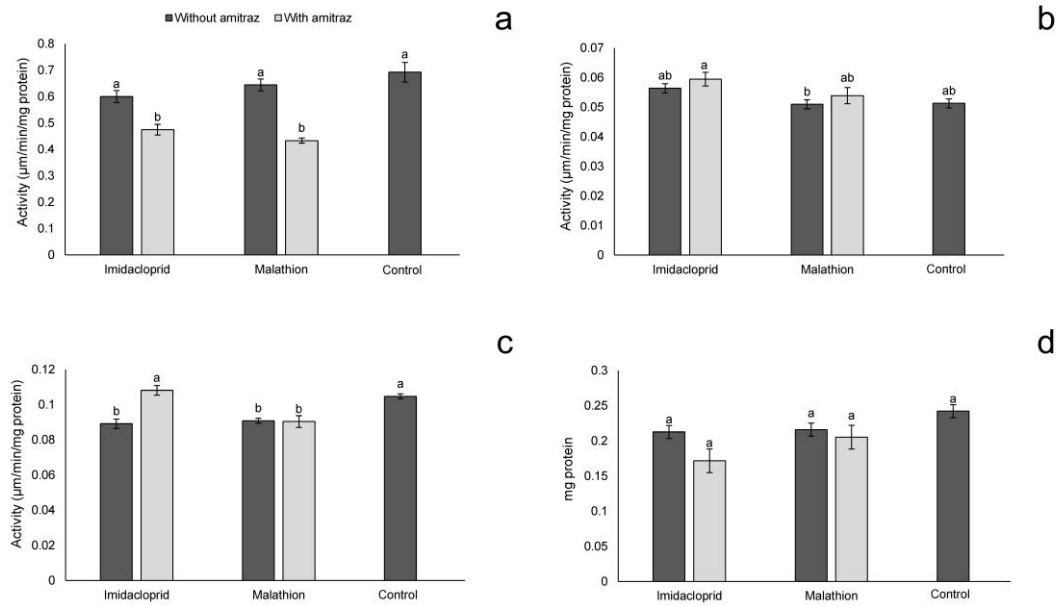
#### Activity of Detoxifying Enzymes

The activity of GST enzymes significantly decreased in aphids treated by a combination of amitraz with each of imidacloprid and malathion. However, the GST activity in aphids treated by each of imidacloprid and malathion (without amitraz) was not statistically different in comparison to the control (Figure 1-a). Combination of amitraz with imidacloprid and malathion did not affect the esterase activity of cotton aphids when 2-naphthyl acetate was used as substrate (Figure 1-b). However, the use of 1-naphthyl acetate as the substrate revealed a significant increase in esterase activity of aphids treated by a combination of imidacloprid and amitraz (Figure 1-c). The

general oxidase activity was not statistically different in aphids treated by imidacloprid and malathion either singly or in combination with amitraz (Figure 1-d).

#### DISCUSSION

In this study, the synergistic interactions between amitraz and two commonly used insecticides, imidacloprid and malathion, were studied on the cotton aphid as target pest. The mortality rates of adult aphids topically exposed to five different concentrations of the insecticides, either singly or in combination with amitraz, were evaluated and the activity of common detoxifying enzymes, glutathione S-transferases, esterases, and general oxidases was quantified.



**Figure 1.** Effect of combination of two insecticides, imidacloprid and malathion, with amitraz on activity of common detoxifying enzymes of the cotton aphids, *Aphis gossypii*, glutathione S-transferases (a), esterases with 2-naphthyl acetate as substrate (b), esterases with 1-naphthyl acetate as substrate (c), and general oxidases (d). Different letters show significant differences at  $P \leq 0.05$ .

Besides reducing the rate of resistance development, pesticide mixtures may enhance the efficiency of control due to synergistic interaction among applied insecticides (Cloyd *et al.*, 2007). Synergism is a well-known phenomenon of chemicals which happens through a variety of mechanisms reviewed in detail by Cedergreen (2014). For example, the synergistic effects of the acaricide and insecticide, amitraz, has been recognized for more than two decades (Horowitz *et al.*, 1987; Liu and Plapp, 1992). Synergistic properties of amitraz in combination with other pesticides, such as fipronil and cypermethrin, have been reported in the control of *Rhipicephalus* ticks (Prullage *et al.*, 2011; Rodriguez *et al.*, 2013). Similarly, Ahmed and Matsumura (2012) showed significant synergistic interaction of amitraz with imidacloprid against *Aedes aegypti*.

Our results confirm the synergistic effects of amitraz on both imidacloprid and malathion. When a non-Lethal Concentration ( $LC_0$ ) of amitraz was used in combination with different concentrations of malathion against cotton aphids, it enhanced aphid mortality at lethal concentrations greater than  $LC_{30}$ . A dose-dependent pattern of synergism was observed in this combination such that the synergism ratio increased with increasing malathion concentrations. Synergistic relationship of amitraz was observed at all lethal concentrations of imidacloprid. In contrast to malathion, synergistic effects of amitraz on imidacloprid decreased by increase in dose; the most intense synergism ratio was observed in aphids treated by a combination of amitraz and  $LC_{10}$  of imidacloprid.

We found a significant decrease in the activity of glutathione S-transferase enzymes of cotton aphids when treated with

a combination of amitraz and each of imidacloprid (a neonicotinoid) or malathion (an organophosphate). When the aphids were exposed to imidacloprid and malathion without amitraz combination, glutathione S-transferase activity was not significantly different with the control aphids that had not been exposed to insecticides (Figure 1-a). These findings may imply that amitraz had synergized insecticidal effects of both imidacloprid and malathion by inhibiting the activity of glutathione S-transferases. These results are in line with some previous studies, which indicated that amitraz could decrease the activity of glutathione S-transferases in arthropods (Da Silva Vaz *et al.*, 2004; Loucif-Ayad *et al.*, 2008).

Despite the detection of a slight increase in esterase activity of cotton aphids which is treated by amitraz combined with imidacloprid and malathion in comparison with the control and treatments without amitraz, the differences among these treatments were not statistically significant. The role of synergists in inhibition of esterases has been highlighted in several studies, nevertheless, our current study may imply that esterase inhibition is not the reason for synergistic interactions which is observed among amitraz and the two studied insecticides in the control of cotton aphids (Sammataro *et al.*, 2005). Similarly, amitraz had no significant effect on the amount of general oxidase of cotton aphids when it was combined with each of malathion and imidacloprid, although, a slight decrease in the amount of oxidase was detected in aphids treated by a mixture compared to aphids treated by single insecticides or the control. The significant increase in the activity of esterase enzymes (Figure 1-c) may be explained by the fact that these enzymes are important agents in detoxifying amitraz.

The main mechanism for amitraz to impose its synergistic effects seems to be the inhibition of glutathione S-transferase

enzymes. More effective synergistic ratios were estimated using higher Lethal Concentrations (LC<sub>40</sub>-LC<sub>90</sub>) of pesticides in combination with amitraz (see Tables 1 and 2). This may imply that combining their higher lethal concentrations may result in better control as well as resistance management of cotton aphid. Additionally, it has been suggested that malathion has low efficacy against cotton aphid, and this pest densities were reported to increase significantly compared to non-treated fields after malathion application (Jones, 2004). The efficacy of malathion against cotton aphid should be enhanced using different methods including the use of synergists. The results of the current study confirm the synergistic properties of amitraz in combination with imidacloprid and malathion against the cotton aphids. In addition, a significant decrease in the activity of glutathione S-transferase enzymes of cotton aphids was observed when treated with a combination of amitraz and each of the two pesticides.

## CONCLUSIONS

The synergistic interactions between amitraz and the two insecticides, namely, imidacloprid and malathion, were studied against *A. gossypii*. The results revealed that amitraz could have a synergistic effect on imidacloprid at different lethal concentrations. The highest synergistic ratio of amitraz and imidacloprid mixture was observed in the lowest dose (LC<sub>10</sub>) of imidacloprid which is appropriate to reduce the cost of cotton aphid control. Moreover, synergistic relationship between amitraz and malathion was detected only at higher concentrations (more than LC<sub>30</sub>). The results of detoxifying enzyme assays showed that the GST activity, as an effective enzyme in the metabolic resistance process, may have been inhibited by amitraz.



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### اثرات سینرژیستی آمیتراز با ایمیداکلوپرید و مالاتیون روی شته جالیز *Aphis gossypii* (Hem: Aphididae)

۱. شجاعی، خ. طالبی جهرمی، و. حسینی نوه، و غ. صباحی

#### چکیده

شته جالیز *Aphis gossypii* از جمله آفات مهم و چند میزبان بوده که بیش از یک قرن است که، مهم‌ترین ابزار کنترل آن، استفاده از آفت‌کش‌های شیمیایی می‌باشد. در حال حاضر، از مخلوط آفت-کش‌هایی با محل تاثیر متفاوت، جهت مدیریت مقاومت این آفت استفاده می‌گردد. در این مطالعه، روابط سینرژیستی آمیتراز با هر یک از دو آفت‌کش ایمیداکلوپرید و مالاتیون مورد بررسی قرار گرفت و از شته جالیز به عنوان حشره مورد آزمایش استفاده شد. همچنین تاثیر آمیتراز بر سطح فعالیت سه آنزیم سم‌زدا در این حشره مورد بررسی قرار گرفت. اثرات سینرژیستی آمیتراز روی ایمیداکلوپرید در کلیه غلظت‌های زیر کشته (LC<sub>10</sub>-LC<sub>90</sub>) مشاهده شد در حالی که، مخلوط آمیتراز با مالاتیون در غلظت‌های بالاتر از LC<sub>30</sub> دارای رابطه‌ی سینرژیستی بود. بیشترین نرخ سینرژیستی در مخلوط آمیتراز با مالاتیون (LC<sub>90</sub>) و ایمیداکلوپرید (LC<sub>10</sub>) به ترتیب ۱/۵ و ۳/۰۹ بود. با توجه به نتایج حاصل از فعالیت آنزیم‌های سم‌زدا، به نظر می‌رسد که علت اصلی در بروز اثرات سینرژیستی آمیتراز با دو آفت‌کش دیگر، به واسطه‌ی کاهش سطح فعالیت آنزیم گلوکاتایون اس ترانسفراز باشد.