EVALUATION OF INSECTICIDE RESISTANCE AND BIOCHEMICAL MECHANISM IN TWO POPULATIONS OF *EURYGASTER INTEGRICEPS* PUTON (HETEROPTERA: SCUTELLERIDAE)

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[Alizadeh, M., Bandani, A. R. & Amiri, A. 2010. Evaluation of insecticide resistance and biochemical mechanism in two populations of *Eurygaster integriceps* puton (Heteroptera: Scutelleridae). Munis Entomology & Zoology, 5 (2): 734-744**]**

ABSTRACT: Sunn pest (*Eurygaster integriceps* Puton) (Heteroptera: Scutelleridae), is one of the most serious insect pests of wheat and barley in Iran and in the neighboring countries. In Iran since 1950's control of Sunn pest was mainly based on chemical control especially of DDT followed by organophosphorus insecticide such as fenitrothion (since 1970's). In the current study susceptibility of the two geographic populations (Mazandaran and Tehran provinces) of the insect to fenitrothion was assessed using bioassays as well as enzymatic evaluations. Calculted LD_{50} values against adult insects of Tehran and Mazandaran populations were 0.19 and 0.08 µg insect^{-1}, respectively. Residual assays against second-instar nymphs of the Sunn pest showed that LC_{50} value for Tehran strain was 1.71 ppm while LC_{50} value for Mazandaran strain was 0.77 ppm. These data indicated that Tehran colony is less susceptible to insecticide than the Mazandaran colony. These findings were further supported by determination of esterase activity and I_{50} values of the two populations. Esterase activity of female individuals of Tehran and Mazandaran colonies to 1-NA (alpha naphthylacetate) substrate was 1.16 and 0.68 µM product/min/mg protein, respectively. The female individuals of Tehran population contained almost twice as much esterase activity as Mazandaran population. Analysis of variance showed that there were significant differences between esterase activity of male and female and between two populations ($P < 0.05$).

KEY WORDS: Sunn pest, Fenitrothion, Susceptibility, Esterase activity.

Sunn pest (*Eurygaster integriceps* Puton) (Heteroptera: Scutelleridae), is one of the most serious insect pests of wheat and barley in Iran and in the wide area of the Near and Middle East, West Asia, and many of the new independent states of central Asia. It also is found in Eastern and South Europe and North Africa (Kazzazi et al., 2005; Radjabi, 2000).

This insect has a monovoltine life cycle (one generation per year) with two different phases. The first phase (growth and development) occurs in the wheat field during the spring, whereas the second phase (diapause as an adult) usually occurs in the mountain area during the summer and winter (Radjabi, 2000). Migration from diapausing site toward wheat field occurs at the end of the winter when the average daily temperature exceeds 10°C. Soon after settling in the field, the insects start feeding followed by oviposition. At 25°C, Embryonic and post embryonic development lasts about 10 and 30 days, respectively.

The Sunn pest feeds on various structures of the host plants e.g. leaves, stems and grains and as a consequence, the nature of the injury they cause is also variable. During feeding they enter their stylets into the host plant, inject their watery saliva which containing digestive enzymes, and sucking out the liquefied cells' contents (Panizzi, 1997; Cohen, 2000; Boyd et al., 2002). Seeds are preferred feeding sites. Feeding punctures appeared as minute darkish spots on

the seeds. Sunn pest feeding on different stages of developing seeds causes quantitative damage because they inject enzymes into the grain that degrade gluten protein and cause rapid relaxation of dough which results in the production of bread with poor volume and texture. The most important times in the life cycle of *E. integriceps* are the period of nymphal development especially after the third instar and the intense feeding of the newly emerged adults before migration. Most of economic loss attributed to this species is caused by larval and adult injury to the wheat grain. Yield loss because of Sunn pest infestation in some area is 100% and it has been observed that because of the insect severe infestation a wide area of wheat field is not harvested. In Iran alone more than one million hectare of wheat field are sprayed against this pest. Spraying area has annually been increased for example in 1976 only 76000 hectares sprayed whereas in 2001 more than one million hectares has been sprayed (Nouri, 2002).

Since 1970's, organophosphorus insecticide fenitrothion extensively used in order to control this insect in Iran. As a consequence of the intensive use of fenitrithion, in recent years there are some complaints about the insecticide performance, especially in areas where spraying was used consistently for different consecutive years. Insects develop resistance to insecticides primarily though three mechanisms: decreased penetration, reduced target site sensitivity and enhanced metabolism (Plapp, 1976; Oppenoorth, 1984). Enhanced metabolism of insecticides decreases the attainment of the effective amount of insecticides that can kill insects. Thus, metabolic resistance may significantly decrease the susceptibility of insects to insecticides. Three major detoxifying enzymes are associated with insecticide resistance: cytochrome P450 monooxygenases, glutathione *S*-transferases and esterases (Bull, 1981; Oppenoorth, 1984).

Esterases are a large group of proteins that use water to hydrolyze endogenous and exogenous esters and produce alcohols and acids as products (Benning, 1994; Kim et al., 1997).

Esterase-mediated metabolic resistance is widespread and has been detected in almost all pests and against all classes of insecticides containing an ester moiety. Enhanced metabolism by esterases is a major mechanism in OP resistance, and has been detected in a number of dipterans (Hemingway, 1981; Whyard et al., 1995), homopterans (Devonshire & Sawicki, 1979; Devonshire & Moores, 1982; Devonshire, 1991), coleopterans (Conyers et al., 1998; Zhou et al., 2002), lepidopterans (Beeman & Schmidt, 1982), and hymenopterans (Baker et al., 1998).

Despite prolonged and extensive use of organophosphorous pesticides against Sunn pest, no documented reports exist on the possible development of insecticide resistance in the insect. So, the aim of the current study was to assess susceptibility of two geographic populations of the Sunn pest to fenitrithion in order to determine occurrence of resistance, if any, in their populations. This knowledge will help to new management strategies for the pest control.

MATERIAL AND METHODS

Insects

The insects were collected from Karaj and Marzan-Abad wheat farms in Tehran and Mazandaran provinces, respectively. The Tehran population was collected from areas where control measures using pesticides have been taking place for more than three decades. Mazandarn population was collected from an

area where control measure is not practiced and assuming this population is susceptible to the pesticide.

The both colonies were maintained and reared on wheat plants and wheat kernels in the laboratory at 25 ± 2 °C under a 14 h light: 10 h dark (LD 14:10) photoperiod.

Topical application bioassays

The efficacy of fenitrothion (EC 50%) against the two different populations of the Sunn pest adults from Tehran and Mazandaran provinces was determined using five concentrations of the insecticide. Fenitrothion were dissolved in acetone to give final concentrations of 0.40, 0.26, 0.17, 0.12, 0.08 µg insect $^{-1}$ for Tehran population while for Mazandaran population working solutions were 0.20, 0.11, 0.06, 0.03, and 0.02 µg insect $^{-1}$. Controls were treated with acetone alone. Bioassays were carried out by topical application of one microliter of insecticide solution to the thoracic notum of adults. Each concentration was replicated four times using 10 adult insects per replicate. All tests were run at 25 ± 2 °C and 14:10 h photoperiod. Mortality was measured at 24 h after treatment and mortality data were corrected using abbot formula (Abbot 1925).

Residual bioassays

Residual assays were done against second-instar nymphs (the same size and age) of the two different of the above mentioned populations. Fenitrothion was dissolved in acetone to give desired concentration. For Tehran population, working solutions of 4.0, 2.0, 1.0, 0.50 and 0.25 ppm were prepared and for Mazandaran population insecticide concentrations of 3.60, 1.3, 0.57, 0.24, and 0.18 were prepared.

Petri dishes (9 cm diameter) lined with Whatman No. 1 filter paper and 800 µl of each concentration were applied to the filter paper followed by air drying for one hour at room temperature. Controls were treated with acetone alone. Each treatment was replicated five times using 20 second-instar nymphs per replicate. All tests were run at 25 ± 2 °C and 14:10 h photoperiod. Mortality was measured at 24 h after treatment and mortality data were corrected using abbot formula (Abbot, 1925).

Enzyme preparation

Preparation of the enzyme was done according to Mendoza et al. (2000) and Barbier et al. (2000) with slight modification. Briefly, the insects (adults) were randomly selected and immobilized by placing them in ice, after which their antenna, legs, wings, and mouthparts were cut and removed. The rest of the insects' bodies were homogenized in ice-cold 0.02 M sodium phosphate buffer (pH 7.0). The crude homogenates were transferred to centrifuge tubes and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant (crude extract) were pooled, stored at -20 °C and used as the enzyme source for subsequent assays.

Enzyme assay

Adults' esterases were determined from both populations according to Van-Asperen colorimetric method (Van-Asperen, 1962), Hemingway and Georgiou (1984) and Mendoza et al. (2000) with some modifications. Esterase activity was measured using alpha- and beta naphthylacetate (Merk, Germany) (1-NA and 2-NA) (4 mg ml^{-1} in acetone). The assay mixture was composed of 20 µl enzyme (containing 5 mg ml^{-1} protein), 260 µl phosphate buffer (0.02M and pH 7.0), 20 µl substrate either 1-NA or 2-NA. The reaction was started by the addition of enzyme and incubation was done in water-bath at 30 °C for 30 min. After that, 65 µl of Fast blue solution (Merk, Germany) (10 mg Fast blue B in 1 ml distilled water plus 5 ml of 5% sodium dodecyl sulfate) was added and left for 10 min to stabilize the color. Then 335 µl phosphate buffer was added to get a final volume of 700 µl

and the absorbance determined at 600 nm by spectrophotometer. Controls were run in parallel, which contained 20 µl of distilled water to replace the 20 µl of the enzyme. All assays were done in duplicates and repeated at least three times. The quantity of naphthol produced from esterase reactions was calculated from standard curves of alpha- and beta-naphthol. Results were expressed as µM product/min/mg protein.

Calculation of I_{50}

To calculate I_{50}s (concentration of the insecticide required to inhibit 50% of the esterase activity) various concentrations of the inhibitor (insecticide) in phosphate buffer were added to the reaction mixture. For Tehran population seven concentrations of 0.0, 38.0, 200.00, 500.0, 950.0, 1400.0, and 1850.0 ppm were used while for Mazandaran population seven concentrations of 0.0, 10.0, 25.0, 40.0, 55.0, 70.0, 85.0, and 125.0 ppm were used. Briefly, each insecticide concentration was pre-incubated with the enzyme at 30 ºC for 30min, followed by addition of the substrate (1-NA) and the assays were proceeded as described in the previous section.

Protein determination

Bradford method (Bradford, 1976) was used to assess protein concentration with bovine serum albumin as standard.

Data analysis

The dose-mortality data from each colony were subjected to probit analysis, using Polo program (Finney, 1971). Lethal dose 50 % (LD_{50}) values obtained in probit were expressed as microgram of fenitrothion per insect. Obtained lethal concentration 50 % (LC_{50}) values were expressed as part per million (ppm). Analysis of variance (ANOVA) was used to compare LD_{50}s, LC_{50}s, and esterase activity within and among populations.

RESULTS

Topical bioassays

Topical assays against both populations of adult insects of *E. integriceps* showed that Mazandaran province insects are more susceptible to the toxic activity of fenitrithion. LD_{50} values were 0.19 and 0.08 µg insect^{-1} against Tehran and Mazandaran populations, respectively (Table 1) (Figure 1). So, based on these LD_{50} values it is evident that Tehran strain is more resistant to insecticide than the other strain. The resistance ratio (R/S) (RR) for resistant population was 2.4 which is relatively low value. Although the resistance ratio is low, there are significant differences between LD_{50} values of the two populations ($F = 80.55$; $P < 0.01$). Also, 95 % confidence limit of two LD_{50} values fails to overlap (Table 1).

Residual bioassays

Residual assays against second-instar nymphs of both populations of the Sunn pest showed that Tehran population has higher LC_{50} value than Mazandaran population (Table 1). LC_{50} value for Tehran strain was 1.71 ppm while LC_{50} value for Mazandaran strain was 0.77 ppm (Figure 2). Analysis of variance showed that there were significant differences between LC_{50} values of the two populations ($F = 7.46$; $P < 0.01$). Calculated resistance ratio (RR) for resistant population was 2.22. Based on LC_{50} values and calculated RR it could be concluded that Mazendaran strain of *E. integriceps* is more susceptible to the insecticide than Tehran strain.

Enzyme activity

Alterations in the esterase activity of the two populations of the Sunn pest were observed. Also, activity of the enzyme between males and females of one population was not the same. The measured activity of esterases from two

populations to 1-NA and 2-NA was summarized in table 2. As can be seen from the table, esterase activity of female individuals of Tehran population to 1-NA and 2-NA was 1.16 and 0.47 µM product/min/mg protein, respectively. Esterase activity of female individuals of Mazendaran population to 1-NA and 2-NA was 0.68 and 0.23 µM product/min/mg protein, respectively. More or less the same values of esterase activity were seen between male individuals of the two populations e.g. esterase activity of males from Mazandaran population against 1-NA and 2-NA was 0.31 and 0.1 µM product/min/mg protein, respectively, whereas esterase activity of males from Tehran population against 1-NA and 2-NA was 0.42 and 0.21 µM product/min/mg protein, respectively. Analysis of variance showed that there were significant differences between esterase activity of male and female and between two populations esterase activity ($P < 0.05$). Also, there were significant differences in esterase activity against two substrates ($P < 0.05$).

I_{50} estimation

The effect of various concentrations of fenitrothion on esterase activity of the two populations was shown in figures 3 and 4. The efficiency of the inhibitor (insecticide) on the two populations was compared based on their I_{50}s. Measured I_{50} for Tehran population was 311.89 µM, whereas calculated I_{50} for Mazandaran population was 40.02 µM. Analysis of variance showed that there were significant differences between I_{50} values of two colonies ($F = 24898.84$, $P < 0.001$) (Fig. 5). Based on these data it should be said that esterase of Mazandaran population was more sensitive to the inhibitory action of fenitrothion than that of Tehran population.

DISCUSSION

In Iran since 1950's and even before control of Sunn pest, *E. integriceps* was mainly based on chemical control especially of DDT followed by organophosphorus insecticide such as fenitrothion (since 1970's) (Javad-Zadeh, 1991). The continuous use of insecticides in some area decreased the susceptibility of Sunn pest population to insecticides. In the current study susceptibility of the two different geographic populations of the insect to the insecticide was assessed using bioassays against both mature and immature stages as well as enzymatic evaluations.

The presented data showed that susceptibility of the sunn pest has changed in the area where insecticides have been used extensively for a long period. LD_{50} values of the Tehran population was 0.19 µg insect $^{-1}$ while that of Mazandaran population was 0.08 µg insect $^{-1}$.These data indicated that Tehran colony is less susceptible to insecticide than the other colony. These findings were further supported by determination of LC_{50} values of pesticide against immature insects. Temizer (1976) reported that LD_{50} value of DDT against Sunn pest has been increased from 0.13 to 0.55 µg insect $^{-1}$ over two year application. Resistance Ratio (RR) for adults and nymphs determined to be 2.4 and 2.2, respectively, which is relatively low. Nevertheless, low resistance ratio might be attributed to restricted generation of insect (one) per year as a result insects are not exposed to the insecticide more than once a year. In some insects RR of more than 2500 have been reported (Baker, 1995). Resistance to deltametrin in Brazilian (RR equal to 7) and Argentinian (RR equal to 2 to 7.9) heteropteran species (*Triatoma infestans*) has been reported (Gonzalez-Audino et al., 2004).

Both Esterase assays and I_{50} estimations confirmed the *in vivo* assays (insecticidal assays) that Tehran colony was less susceptible to the action of fenitrothion insecticide. Fenitrothion is organophosphorus insecticide which is

susceptible to hydrolysis and sequestration by esterases. The enzymes that hydrolyze simple ester substrates such as naphthylacetate are termed non-specific esterases or general esterases. The esterase abundance protects the resistant insects by binding and sequestering insecticides rather than by rapid hydrolysis (Haubruge et al., 2002). Esterases are divided into three groups depending on their inhibition by organophosphate: arylesterase (A type), carboxylesterase (B type) and acetyl esterase (C type) (Barbier et al., 2000). Elevated esterase activity has been implicated in resistance to insecticides of a variety of insects (Devonshire & Sawicki, 1979; Hemingway, 1981; Devonshire & Moores, 1982; Mouches et al., 1986; Argentine et al., 1989; Devonshire, 1989; Whyard et al., 1995; Baker et al., 1998; Haubruge et al., 2002; Wang et al., 2004). In the current study it was found that esterase activity of female individuals of Tehran and Mazandaran colonies to 1-NA substrate was 1.16 and 0.68 μM product/min/mg protein, respectively. So, the female individuals of Tehran population contained almost twice as much esterase activity as Mazandaran population. For example in various *Tribolium* strains, the naphthyl esterase activity was much more active in susceptible strain than in malathion resistant strain (Wool et al., 1982; Haubruge et al., 2002).

Our results showed that female individuals have more esterase activity than males. For example in Tehran population esterase activity of females determined to be about 2-fold more than males (Table 2.). While the reason/s for differences is not known, it could be possible attributed to the high content of fat body in the female than male individuals. Our unpublished data indicated that females are heavier than males e.g. well feed females usually weigh more than 100 mg while males in the same population weigh less than 100 mg. The other reasons that could be said are the searching of females for oviposition sites. It has been shown that selection by toxic substances can increases the amount of enzymes that are responsible for detoxification (Ferrari & Georghiou 1990; Scharf et al., 1998; Mendoza et al., 2000). The females may have been under continual selection pressure when seeking plant fluids, resting sites or oviposition sites that are associated with insecticide-treated area. Thus, these types of selection pressure may have played a significant role in the higher esterase activity observed in the females. It has been reported that gene amplification is involved in the increase of enzyme quantity. Also, gene amplification reported to be the mechanism causing protein overproduction when an organism is under environmental stress (Mouches et al., 1990).

Based on the values of I_{50}s, Tehran colony of Sunn pest was less sensitive to the inhibitory action of fenitrothion than that of Mazandaran population. The differences are due to the different geographic population used that Tehran population has been more under selection pressure than that of Mazandaran.

The presented data suggest that the higher sensitivity of Mazandaran population to insecticide than Tehran population implies that underlying mechanism of tolerance in insecticide bioassays is largely due to metabolic activities which either detoxify or limit the intoxicating ability of fenitrothion. So, the present study has provided some basic information on the esterases of these two populations that will be useful to understand the mechanisms of insecticide resistance in the *Eurygaster integriceps*.

ACKNOWLEDGEMENTS

This work was funded by a grant from University of Tehran.

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Table 1. The toxicity of fenitrothion to E. *integriceps* adults and second instar nymphs of two different geographic populations (Tehran and Mazandaran provinces) of Iran.

Insect	Population	LD_{50} (µg/insect) for adults and LC_{50} (PPM) for nymphs and their 95% confidence limit[1].	Slope ± SE	X^2	dF
Adult	Tehran	0.190 (0.169-0.222)	3.313±0.218	1.140	3
	Mazandaran	0.083 (0.070-0.103)	2.480±0.123	0.577	3
Nymph	Tehran	1.710 (1.339-2.385)	1.832±0.091	2.568	3
	Mazandaran	0.766 (0.600-0.995)	2.310±0.555	3.317	3

1. LD_{50} or LC_{50} is considered significantly different when the 95% confidence limit fails to overlap.

Table 2. Esterase activity of two different geographic populations (Tehran and Mazandaran provinces) of *E. integriceps* to 1-NA (alpha-naphthyl acetate) and 2-NA (beta naphthyl acetate).

Population	Substrate	Sex	Esterase activity (µM product/min/mg protein)
Tehran	1-NA	Male	0.47 ±
		Female	1.16 ±
	2-NA	Male	0.21 ±
		Female	0.42 ±
Mazandaran	1-NA	Male	0.31 ±
		Female	0.68 ±
	2-NA	Male	0.1 ±
		Female	0.23 ±

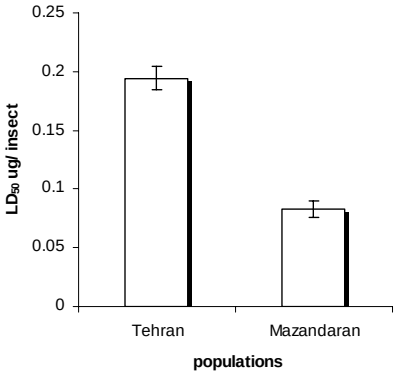

Figure 1. Comparison of toxicity (LD_{50} values) of the fenitrothion against adults of Tehran and Mazandaran populations of the Sunn pest.

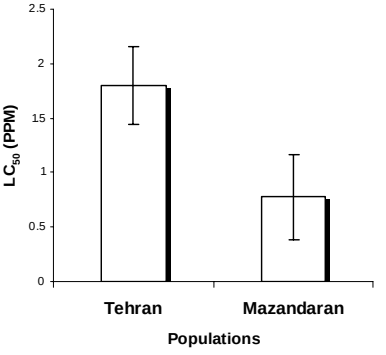

Figure 2. Comparison of toxicity (LC_{50} values) of the fenitrothion against second instar nymphs of Tehran and Mazandaran populations of the Sunn pest.

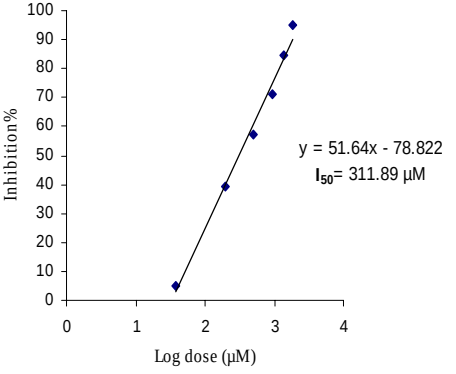

Figure 3. Inhibitory effect (I_{50}) of fenitrothion on adult esterase activity of Tehran population.

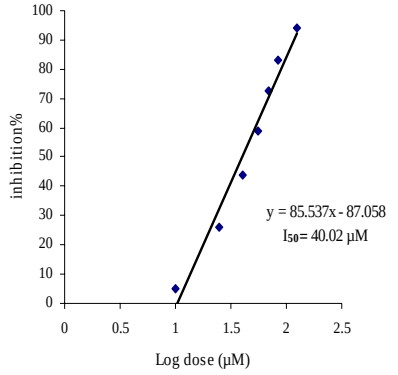

Figure 4. Inhibitory effect (I_{50}) of fenitrothion on adult esterase activity of Mazandaran population.

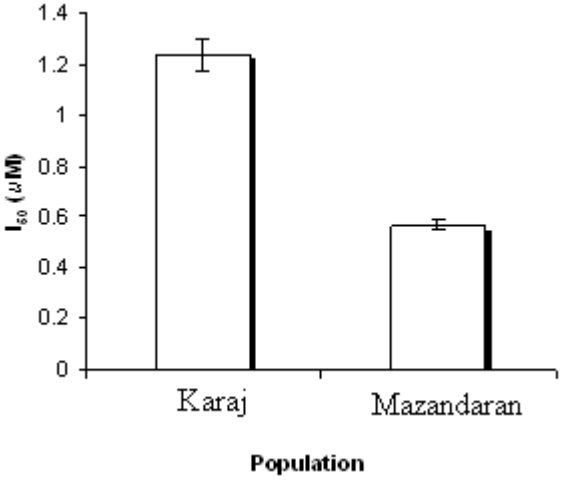

Figure 5. Comparison of toxicity (I_{50} values) of the fenitrothion to esterase activity of the adults of Tehran and Mazandaran populations of the Sunn pest.