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# Insecticide Resistance in Bactocera zonata (Diptera: Tephritidae) in District, Sargodha, Pakistan

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## Abstract

Present study was designed to investigate the role of non-specific esterases, glutathione-S-transferesaes and monooxygenases in insecticide resistance in *Bactocera zonat.* Flies were collected from Gauva orchids of Ajnala, Sargodha. For biochemical estimation of detoxifying enzymes, flies were exposed to the selected insecticides (i.e. Trichlorofon, Malathion and λ-cyhalothrin) for one hour, and then shifted to clean jars. The activity of insecticide detoxifying enzymes in the survivors and control were compared. The activity of insecticide detoxifying enzymes was higher in survivors compared to the susceptible flies (control). The activity of beta esterases, glutathione S-transfereases and monooxygenases of Trichlorofon and Malathion treated flies did not differ. However, both groups have higher activities of detoxifying enzymes compared to the control. Higher activities of insecticide detoxifying enzymes in survivors compared to the control groups indicate the involvement of these enzymes in insecticide resistance.

**Keywords:** Esterases; Glutathione S-transferases; Monooxygenases

## Introduction

Fruit flies (Diptera: Tephritidae) are one of the most economically important groups of horticulture insects pests worldwide [1,2]. They contribute loss of millions dollar to fruits and vegetable marketing in the region. In Pakistan, synthetic insecticides of different chemistry and mode of action are being used for the management of fruit flies [2,3]. Long term and extensive use of insecticide of same chemistry and mechanism, not only cause resistance among insect pests, but also put stress on the economy of the country. Resistance against many insecticides (i.e. DDT, methoxychlore, malathion, bifenthrin, trichlorfon, lamda cyhalothrin and sipnosad) has been well studied in many Tephritidae pests [2]. In Pakistan, resistance against diptrex (Trichlorofon), Malathion [4], bifenthrin and λ-cyhalothrin has been reported in *Bactocera dorsalis* and *B. zonzta* [2]. There are many physiological mechanisms for insecticide resistance, but increased metabolic detoxification and decreased target site sensitivity are considered important [5].

Esterases are the most significant enzymes, involved in the metabolism of insecticides [6]. Carboxylester and phosphodiested bonds of carbamates, organophosphate and pyrithroids are attacking sites for esterases [7,8]. Esterases are able to break an ester bond by using water molecule and cause insecticide resistance, either by metabolizing very restricted range of insecticides containing ester bonds, or by rapid-binding and sequestration of insecticides. Glutathion S-transferases belong to a vast family of multifunctional interacellular enzymes, which are involved in detoxification of both endogenous and exogenous chemicals [9-11]. Involvement of one or more GST in resistance to organophosphate is reported in house-fly *Musca domestica* [12], to organochloride in fruit fly *Drosophila melanogester* [13], and to pyrithroids in planthopper *Nilaparvata lugens* [14]. These enzymes also protect the body tissues from oxidative stress [9].

Monooxygenases are phase I metabolic enzymes [15]. They metabolize a wide range of insecticides by hydroxylation, epoxidation and oxidation [16]. They also play a vital role in tolerance of plant toxins in insects [17]. Generally, monooxygenases are found in all body tissues, but highest activities are observed in mid gut. Monooxygenases are mainly involved in the metabolism of pyrethroids, and to a lesser extent in the detoxification of organophosphates and carbamates [16]. In *B. dorsalis,* increased activity of monooxygenases is found to be associated with malathion resistance, along with carboxylesterases [6]. Over expression of genes is recognized as chief mechanism of monooxygenase based resistance. The over expression of the P450 enzymes in pyrethroid resistant insects is well documented [18].

As studies have revealed a positive correlation between levels of detoxifying enzymes and insecticide resistance, so quantification of these enzymes is used to monitor resistance against insecticides, and will be helpful in future for the better management of insect pests in the area. Objective of current study was to determine the susceptibility status *Bactocera zonata* in the study area, and also to quantify and compare the levels of insecticide detoxifying enzymes in the control and flies exposed to different insecticides.

## Materials and Methods

### Test organism-sampling

Test insects were obtained from Ajnala, Sargodha (30°10" N and 72°45" E). Adult fruit fly (*Bactocera zonata*) were collected from fallen infected fruits and brought to the laboratory. These were reared in 2×2×2 ft plastic cages under controlled conditions (Photo period 12:12 (L: D); temperature, 26 ± 2°C; Relative Humidity 60 ± 5%). Three to four day old adults were used in the experiment.

### Enzyme preparation

For biochemical estimation of detoxifying enzymes, flies were exposed to the selected insecticides (i.e. Trichlorofon, Malathion and λ-cyhalothrin) for one hour, and then shifted to clean jars. After 24 hours of exposure, survivors were frozen at -20°C for 15-20 minutes in

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order to immobilize the insect, and then wings, legs and abdomen of flies were removed. Rest of the body was homogenized in 400 µl chilled phosphate buffer (0.1 M, pH 7.0), containing 0.01% (w/v) of triton X-100 [19]. The crude homogenate was centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was collected and used further as enzyme source for biochemical estimation of non-specific esterases (β-esterases), glutathione s-transferases (GST), and mixed function oxidases (MFOs). Unexposed flies were used as control.

### Biochemical estimation of non-specific esterases

To measure the activity of non-specific esterases, β-naphthyl acetate was used as substrates [20]. The reaction mixture contained 20 µl homogenate, 20 µl substrate solution (0.1 M) and 150 µl phosphate buffer (100 mM). The reference solution of reaction mixture contained 20 µl substrate solution and 170 µl phosphate buffer. These mixtures were incubated for 30 minutes at 37°C. When incubation time was over, 100 µl of mixture of 1% fast blue b (FBB) salt and 5% sodium dodecyl sulphate (SDS) in ratio 2:5 was added to stop the reaction. After 15 minutes optical densities were recorded at the wavelength of 545 nm using microplate reader (Thermo Scientific Multiskan FC Microplate Photometer). The optical densities (OD) were compared with standard curve of β-napthol, in order to convert the absorbance into product concentrations. The enzyme activities were expressed as mmol of product formed/min/mg of protein.

### Biochemical estimation of glutathione-s-transferases

The activity of Glutathione S-trasferases towards 1-chloro-2, 4-dinitrobenezene (CDNB) was estimated according to the method of Habig et al. [21]. The reaction mixture was comprised of 40 µl 1.0 mM reduced Glutathione, 20 µl 1.0 mM 1-chloro-2, 4-dinitrobenzene (CDNB), 200 µl phosphate buffer (100 mM, pH 7.0), and 20 µl of supernatant. Reference solution for reaction mixture contained 20 µl 1.0 mM CDNB, 200 µl phosphate buffer (100 mM, pH 7.0), and 40 µl 1.0 mM reduced Glutathione. The absorbance was measured at 340 nm for 5 minutes, after five minutes of the reaction. Change in the absorbance per minute was converted into nano-moles of CDNB conjugate/min/mg of protein, by using extinction coefficient (ε) of 9.6 mM $cm^{-1}$ for CDNB-GSH conjugate.

$$\text{CDNB}-\text{GSH conjugate formed}\left(\text{nM}/\text{mg of protein}/\text{min}\right)=\frac{\text{AB}/\text{s}\left(\text{change in 5 min}\right)\times 0.28\times 1000}{9.6\times 0.5\left(\text{path length}\right)\times \text{protein in mg}}$$

### Biochemical estimation of monooxygenases

Reaction mixture comprised of 20 µl of supernatant, 200 µl of 3,3, 5',5'-Tetramethyl benzidine (TMBZ) solution (TMBZ solution was made by dissolving 0.01 g TMBZ in five ml ethanol and 15 ml 0.25 M sodium acetate buffer (PH 5.0)), 100 µl of 0.625 M potassium phosphate buffer (PPB) at pH 7.0 and 30 µl of 3% hydrogen peroxide. Reference solution for reaction mixture contained 200 µl TMBZ, 120 µl, 0.625 M potassium phosphate buffer (PPB) at pH 7.0 and 30 µl 3% hydrogen peroxide. After ten minutes readings were recorded at the wavelength of 620 nm [22]. The quantity of monooxygenases was calculated from a standard curve of cytochrome C.

### Statistical analyses

One way ANOVA followed by Tukey's test was used to compare enzyme activities against different insecticides. All statistical analyses were performed using SPSS (version 13).

## Results

The activity of esterases in insecticide treated male flies was significantly different from control group (df=3, 42; F=456.96; P>0.001). Results of Tukey's test showed that there was no difference in esterase level in male flies treated with Trichlorofon and Malathion, but both groups differ significantly from control group (Table 1). It is also depicted in the table 1 that the level of esterases in λ-cyhalothrin treated flies was not only different from control group, but also from Trichlorofon and Malathion treated flies. When four groups of female flies were compared for beta esterase levels, we found statistically significant difference (df=3, 44; F=266.67; P=0.001). Trichlorofon and Malathion treated flies showed higher esterase activity compared to the control (Table 1).

Glutathion S-Transferase activities were also significantly different among different group of male flies (df=3, 42; F=214; P>0.001) (Table 2). Results of Tukey's test showed that GST activity was higher in Malathion and λ-cyhalothrin treated flies, while Trichlorofon treated flies did not differ significantly from control and Malathion exposed flies. The activities of GST activity among female flies also vary significantly (df=3, 44; F=18.26; P=0.001). Activities were higher in Malathion and λ-cyhalothrin exposed flies than control group, respectively, while Trichlorofon treated flies did not differ from control and λ-cyhalothrin treated flies.

The activity of moooxygenases were also significantly different among different groups of male and female flies (df=3, 42; F=3.54; P=0.02 for male flies and df=3, 44; F=3.94; P=0.02 for female flies) (Table 3). According to Tukey's test, among male flies, enzyme activity was significantly high in Trichlorofon and λ-cyhalothrin treated group of flies, as compared to control group while levels of GST activity in Malathion exposed group do not differ from any group, expect λ-cyhalothrin treated group of flies. In female flies, significantly higher enzyme activities were recorded in λ-cyhalothrin treated group.

## Discussion

Participation of enzyme such as specific or non-specific esterases, monooxygenases and transferases has long been documented in insecticide detoxification and resistance development [16]. Our results were in accordance with that of Hsu et al. [6], who has reported increased activities of esterases towards α-napthyl acetate and β-napthyl acetate, and higher activities of mixed function oxidesases in malathion resistant strains of *B. dorsalis* than that of susceptible strains. While in case of GST, our findings were contrary to Hsu et al. [6] in a way that there was non-significant difference in enzyme activity between resistant and susceptible strain. In Malathion exposed populations, slightly elevated activity of β-esterases and Monooxygenases than unexposed groups, may explain the association of these enzymes with degradation of Malathion in insect body. Quantitative increase in esterases has also been documented relative to malathion resistance in sheep blowfly [23],

| Treatment | Males | Females |
|---|---|---|
| Control | 226.6 ± 27.19$^{a}$ | 311.96 ± 41.21$^{a}$ |
| Trichlorofon | 426.26 ± 39.21$^{b}$ | 573.12 ± 89.29$^{c}$ |
| Malathion | 470.16 ± 31.46$^{b}$ | 560.61 ± 34032$^{c}$ |
| λ-cyhalothrin | 505.19 ± 41.49$^{c}$ | 427.71 ± 48.57$^{b}$ |
| | **Df**=3,42<br>**F**=456.94<br>**P**>0.001 | **df**=3,44<br>**F**=266.67<br>**P**>0.001 |

**Table 1:** Results of Tukey's test comparing activity of β esterases in male and female *Bactocera zonata* exposed to different insecticides.

| Treatment | Males | Females |
|---|---|---|
| Control | 0.64 ± 0.12[a] | 0.7 ± 0.20[a] |
| Trichlorofon | 1.14 ± 0.11[ab] | 1.29 ± 0.16[ab] |
| Malathion | 1.79 ± 0.21[b] | 1.9 ± 0.37[c] |
| λ-cyhalothrin | 2.27 ± 0.21[c] | 1.69 ± 0.16[b] |
| | **Df**=3,44<br>**F**=214<br>**P**>0.001 | **df**=3,44<br>**F**=18.26<br>**P**=0.001 |

**Table 2**: Results of Tukey's test comparing activity of Glutathion S-transferases in male and female *Bactocera zonata* exposed to different insecticides.

| Treatment | Males | Females |
|---|---|---|
| Control | 1.03 ± 0.11[a] | 1.2 ± 0.12[a] |
| Trichlorofon | 1.49 ± 0.07[b] | 1.29 ± 0.2[a] |
| Malathion | 1.18 ± 0.13[ab] | 1.39 ± 0.1[a] |
| λ-cyhalothrin | 2.87 ± 0.009[c] | 1.89 ± 0.13[b] |
| | **Df**=3,42<br>**F**=3.54<br>**P**=0.02 | **Df**=3,44<br>**F**=3.94<br>**P**=0.02 |

**Table 3:** Results of Tukey's test comparing activity of Monooxygenases in male and female *Bactocera zonata* exposed to different insecticides.

*Ceratitis capitata* [24], and petromalid parasitoid [20]. Hsu et al. [6] also reported involvement of esterases in Trichlorofon and Malathion resistance. It is believed that monooxygenases and esterases are involved essentially equally in insecticide detoxification. It is thought that monooxygenases first degrade malathion into malaoxon, a highly toxic compound, which can then be easily attacked by esterases.

Hemingway et al. [25] explained that GST carries out O-dearylation and O-dealkylation processes, in order to detoxify organophosphate based insecticides. According to findings of Hsu et al. [6], only esterases are involved in resistant *B. dorsalis* against Nalid, Trichlorofon and Malathion. In contrast, according to Vontas et al. [14], there is no major role of esterases, GST and monooxygenase activity in organophosphate detoxification in *B. oleae.*

In our study increased β-esterases, GST and monooxygenases activities in Trichlorofon exposed populations were observed. GST is involved in pase II detoxification of Pyrithriod [26] and organophosphate [27], in *Ades agepti*. In *B. dorsalis,* Hu et al. [28] also documented the involvement of GST in pyrithroid (β-cypermethrin) detoxification. In *Anophelese funestus*, pyrithroid resistance may be due to mixed function oxidases (MFO), along with GST based detoxification as secondary mechanism [29]. Involvement of monooxygenases in pyrethroid resistance in many mosquito species is documented as well by Hemingway and Ranson [7]. Vontas et al. [14] has reported GST based detoxification of pyrithroids in *Nilaparvata lugens* and Kostaropoulos et al. [30] in *Tenebrio molitor*. According to Chen et al. [31] in insecticide degradation, GSTs are of much less importance in adult *B. minax* than that of larvae. Our results, which showed slightly elevated activities of Monooxygenases activities in λ-cyhalothrin exposed populations, were in accordance with the findings of Chareonviriyaphap et al. [32], who reported increased activities of monooxygenases in pyrithroid (deltamethrin) resistant *Anopheles minimus* in Thailand. But in case of β-esterases, our results were contrary to him. Involvement of esterases, followed by mixed function oxidases, is also documented in pyrithroid resistance among different insects [33,34].

Involvement of other detoxifying mechanisms like altered target site sensitivity [35-38], mutations in genes driving detoxifying machinery [39,40], decreased penetration [41,42], and behavioral modifications to escape contact to toxins [43,44], are such phenomenon which are not considered in present study, but documented by many scientists. In future, we will extend our scope of study and investigate the involvement of other mechanisms of insecticide resistance.

Our results suggest that, as yet the insecticide resistance in *B. zonata* has not developed, but marked increase in levels of detoxifying enzymes after insecticide exposure in the flies compared to the control group strongly suggests the involvement of studied enzymes in the resistance mechanism.

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