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EFFECT OF PESTICIDES ON GLUTATHIONE S-TRANSFERASE ACTIVITIES IN FORAGER WORKER BEES OF *APIS MELLIFERA* L.

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ABSTRACT : The effect of sublethal concentrations (1/8 and 1/4 of LD_{50} at 24 hrs.) of four pesticides including three *organophosphates*, Dimethoate, Malathion and Quinalphos and one *biopesticide* Neem oil was evaluated on Glutathione S-Transferase (GST) activity in forager worker bees of honeybee *Apis mellifera* L (*A. mellifera* L). The specific activities of GST were measured in forager bees after 24 hrs of treatment to explore the possible changes in proposed enzyme activity under stress of pesticides. Our results indicated that treatments with organophosphates led to increased GST activity in forager worker bees as compared to control group but neem oil, a biopesticide had no toxic effect. The observations based on treated groups indicated that Dimethoate was most toxic as it induced the maximum increase in GST activity followed by Malathion and Quinalphos respectively at sublethal concentrations. The increase of GST indicated that bees were exposed to toxic stress when organophosphates were used against insect pests. There was significant increase in GST activity at concentration-2. Neem oil was found to be nontoxic as it caused no significant change in GST activity.

Key words : *Apis mellifera*, pesticides, organophosphates, foragers, Glutathione S-Transferase (GST), biochemical analysis.

INTRODUCTION

A large number of pesticides are being used in crop fields against the different types of pests all over the world. Unfortunately pesticides are not selective to pest species alone. Often non-target insects such as honeybees, which are economically important, are destroyed in the process of pest control (Atkins *et al,* 1975). While pest insects are the main targets of manufactured insecticides, non-target organisms such as pollinators may come under their attack affecting about 35 % of the world food crops (Velthuis and van Doorn, 2006). The adverse impact that broad-spectrum pesticides have on non-target beneficial insects is widely known to be a major cause of pollinator decline in cultivated areas (Blacquiere *et al*, 2012; Costa *et al*, 2014; Van Engelsdorp and Meixner, 2010). There is no doubt that honey bees are the prominent and economically most important group of pollinators worldwide. Therefore, there is a great concern about the decline of the honey bee population (*A. mellifera* L.) in several parts of the world mainly due to improper application of pesticides (Van Engelsdorp and Meixner, 2010). The pesticides kill bees during some stage of development (like simple poison) or it affects developmental process in such a way that abnormalities are visible in pupa or adult (amorphogenic action) (Johnson *et al*, 2010). Honey bees are of particular interest because they come to contact with various pollutants during their foraging activity, making them a perfect bioassay agent for monitoring pesticide toxicity in urban and rural areas (Porrini *et al*, 1996; Smith and Wilcox, 1990). There are several methods to measure the efficiency of pesticides, mainly against their targets which are also applicable for the non-target organisms. Biomarkers have been used to reveal the exposure of organisms to various chemicals in the environment (Hyne and Maher, 2003). Enzymes are commonly used as biomarkers or indicators for the spread of environmental pollutants such as pesticides and heavy metals. Acetylcholinesterase (AChE), carboxylesterase and glutathione S-transferase (GST) are examples of these enzymes that can be assayed to indicate any behavioral and functional changes in both target and non-target insects exposed to high or sublethal doses of pesticides (Bandyopadhyay, 1982; Guilhermino *et al,* 1998; Kumar and Gupta 2007; Tu *et al*, 2009) and some pyrethroids (Badiou *et al*, 2008). Other classes of environmental contaminants such as complex mixtures of pollutants, detergents and metals are also involved in AChE reduction (Frasco *et al*, 2005; Payne *et al*, 1996). AChE depression has been widely used as a biomarker of general exposure to pollutants, especially organophosphate and carbamate pesticides (Tu *et al*, 2009). However, the activities of other enzymes such as GSTs and carboxylesterase involved in the detoxification

and removal of a wide variety of toxic compounds by conjugation or hydrolysis are also monitored (Hinton *et al*, 1995). Information on the toxicity of pesticide doses used in the agricultural crops in India or other part of the world on honeybee *A. mellifera* L. is very important. Therefore, the aim of the present research is to determine the respective toxicity of Organophosphates Dimethoate, Quinalphos, Malathion, and one biopesticide Neem oil on GST activity as a biomarkers of environmental stress in *A. mellifera* L.

MATERIALS AND METHODS

Pesticides

Sublethal formulations of all the organophosphate pesticides and neem oil, a biopesticide were used for this study. The sublethal concentrations of all pesticides were determined by acute toxicity assay tests. The detail of the pesticides pursued for the experiments includes Dimethoate (Organophosphate – 30 EC, subtethal conc. 0.0075% as Conc-1 and 0.0150% as Conc-2 supplied by Bayer Ltd, India), Quinalphos (Organophosphate – 25 EC, subtethal conc. 0.01% as Conc-1 and 0.020% as Conc-2 supplied by Gujrat Pesticides Limited, India) and Malathion (Organophosphate – 50 EC, subtethal conc. 0.0250% Conc-1 and 0.0500% Conc-2 supplied by Bayer Ltd, India), and *one biopesticide* Neem oil – 25 EC, 0.050% Conc-1 0.10% Conc-2 supplied by Ozonbiotech Ltd, India.

Worker honey bees

The laboratory experiments were conducted with worker honey bees of *Apis mellifera* L (Hymenoptera: Apidae) in Zoology Laboratory of Govt PG College, Bisalpur, Pilibhit. The adult worker bees were obtained from nearby apiary established by Govt Horticulture Department, Bareilly, India where honey bee colonies were maintained according to the standard commercial technique in the field. For this kind of risk assessment, worker honeybees are considered the most ecologically relevant when they start performing external tasks (Picard-Nizou *et al*, 1995). Extensive literature confirms that foragers are those higher than 20 days of age in a typical colony of honey bees (Winston, 1987). Based on farming records, no obvious diseases were observed on units or colonies, and no hives were treated with pesticides. This was confirmed during the collection of bees. Foraging workers were collected as explained by Iwasa *et al* (2004). Briefly, four hives were exposed to smoke twice for 30–60 s before collection. Worker honey bees were collected by shaking from the top super or from the front of the hives into a clean and large plastic container. The container was covered with a solid lid, kept in good condition, and transported to the laboratory in 1 hour. The bees were kept in experimental cages (10×7×12 cm) in groups of 50 at 30 ±2°C with 62 ±5% RH, and fed a 50% (w/v) sucrose solution.

Pesticide treatment

The acute toxicity of the pesticides was evaluated on foraging workers of honey bees (*A. mellifera* L.) by oral administration through sugar syrup application under controlled laboratory conditions. The worker bees were orally treated with aforesaid pesticides with different concentrations in 50% sugar syrup. Prior to treatment with insecticides, bees were anesthetized by cooling (4°C for no longer than 3 min) for handling during bioassay techniques. Each treatment of each concentration was composed of three replicates of plastic cups of 25 bees each covered with a nylon mesh with 75 honeybees total/ treatment (three replicates with 25 bees/cup). The amount of insecticide solution (20 ml) was applied on a cotton bed and then attached to the upper surface of the nylon mesh cover of each cup (three replicates per each concentration) and bees were left to feed for 24 h by lapping from the fibers of the cotton wool. The control group of Bees was fed with 50% (w/v) sucrose solution. The tests were carried out at 30 ±2°C with 62 ±5% RH.

Biochemical studies

Specific activities of GST were determined in midgut of foragerbees of *Apis mellifera* L. GST was extracted from the midgut of tested bees. Surviving bees were anesthetized by cooling at 4 °C for 3 min and the midguts were obtained by pulling the sting from honey bees and were then weighed and rinsed in ice-cooled phosphate buffer (pH 7.0). To measure the activity of the aforementioned enzyme, crude extract of assigned tissue was used. For crude extract, the particular tissue was collected from the exposed foraging bee workers, weighed, and mixed with the proper volume of extraction mixture to make a 10% (w/v) extract. The extraction solution consisted of 10 mM NaCl, 1% (w/v) Triton X-100, and 40 mM sodium phosphate buffer (pH 7.4). The tissues were homogenized in the extraction solution using a glass/ Teflon homogenizer on ice. The homogenate was filtered through cheesecloth and centrifuged at 10,000 rpm (Rami, India) for 20 min at 4°C. The tissue pellet was subjected to extraction and centrifugation thrice with phosphate buffer (pH 7.4). The recovered supernatant fraction containing crude enzyme was mixed and used immediately for assaying GST. All procedures were carried out at 4°C, and all of the experiments were performed in triplicate. GST activity was measured as described by Saint-Denis *et al* (1998) by mixing of 1,650

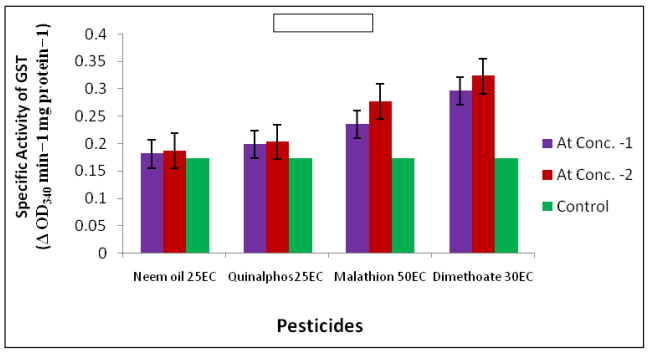

Fig. 1 : Showing GST activity (ÄOD$_{340}$ min^{-1} mg protein^{-1}) in forager bees of *A. mellifera* L.

over control group of bees but concentration-2 caused significant increase (P <0.05) in GST activity proving very toxic to forager bees. The most significant increase was observed in dimethoate treated bees where specific activity increased 0.323 and 0.296 at conc.-1 conc.- 2 respectively. This trend was followed by Malathion, quinalphos as shown in table 1. It can be concluded that GST activity was strongly increased by Dimethoate followed Malathion, Quinalphos, and then neem oil. Neem oil, a biopesticide caused insignificant changes in in GST activity.

Table 1 : Increase in GST activity at sublethal conc-1 and conc-2 of pesticides in midgut of forager worker bees of *A. Mellifera* L. using three replicates. Data are expressed as means ± S.E. Asterisks indicate a significant difference with the control (P ≤ 0.05). Values in parenthesis are % increase in activity of enzyme in treated bees over control bees.

Treatment	Specific Activity of GST (Δ OD$_{340}$ min^{-1} mg protein^{-1})		
	At Conc. -1	At Conc. -2	Control [Sugar Syrup (50%)]
Neem oil 25EC	0.181 ±0.051(4.62)	0.187 ±0.056(8.09)	0.173 ±0.042(—-)
Quinalphos 25EC	0.198 ±0.062(14.45)	0.203* ±0.065 (17.34)	0.173 ±0.042(—)
Malathion 50EC	0.235* ±0.073 (35.83)	0.277*±0.081(60.11)	0.173 ±0.042(—)
Dimethoate 30EC	0.296* ±0.080(71.09)	0.323* ±0.0917(86.71)	0.173 ±0.042(—)

*OD means Optical Density.

µL of 100 mM phosphate buffer (pH 7.4) containing 1m MEDTA, 50 uL of enzyme extract, 200 µLof 2.5 mM reduced L-glutathione (GSH, Merck, India), 100 µL of 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB, Merck, India) as a substrate. The absorbance was measured at 340 nm using single beam spectrophotometer (Systronics 166 Model). One unit of activity corresponded to the quantity of enzyme conjugating 1 mmol of GSH per min. The specific activity was expressed as Δ OD340 min^{-1} mg protein^{-1}.

Statistical analysis

Statistical analysis was performed using the Microsoft office version 2013 Excel spread sheet in Window 7 and minitab 2010 version 13. Means and standard error (SE) were determined from three independent replicates of each treatment. The log dose–response curves were used for the determination of LC$_{50}$ and LD$_{50}$ values for the insect bioassay according to probit analysis (Finney, 1971). All data of GST activities were analyzed by one way analysis of variance (ANOVA) and statistical significance was investigated with Student's t-test at p ≤ 0.05.

RESULTS

The results presented in Table 1 (Figs. 1 and 2) revealed that the specific activity of GST in the untreated bees (0.173) was lower than that of the treated bees at all tested concentrations. Concentration -1 of pesticides (excluding neem oil) increased GST activity insignificantly

DISCUSSION

There are a large number of insects which are economically very important to human being and environment so the study of the effects of pesticides on insects requires measurement of reasonable and appropriate parameters quantitatively. Unfortunately, there has been little work in insects examining biomarkers for sublethal exposure to pesticides. Honey bees are one of the few types of insects where pesticide-related death incidents are neither intended nor welcomed. According to Hyne and Maher (2003) and Kumar and Gupta (2007) bees may proceed as a reliable biomarker for environmental pollution through their reduced pollination capability, the presence of pesticides residues in their honey, their mortalities and enzyme inhibition in their tissues when exposed to lethal rates of pesticides. Under laboratory conditions, the field recommended doses of pesticides are proven to kill foragers, while sublethal concentrations of pesticides have adversely affected colony function (Decourtye *et al*, 2005; Smirle *et al*, 1984). Insecticide actions on the mortality and biochemistry of honey bees have been the subject of many studies (Atkins *et al*, 1981; Costa *et al*, 2014; Decourtye *et al*, 2005; Mayer and Lunden, 1986; Mayer *et al*, 2001; Porrini *et al*, 1996; Rabea *et al*, 2010). The responses of some biochemical parameters, such as alkaline phosphatase, acetylcholinesterase, and glutathione-S-

transferase have been extensively characterized in laboratory studies after the exposure of honey bees to various chemicals (Badiou-Bénéteau *et al*, 2008; Kumar and Gupta, 2007). According to the toxicity of pesticides on honey bee *A. mellifera* to their response profiles to chemicals, honey bees can be considered promising tools for environmental biomonitoring programs. In the present study, the response of GST was evaluated in midgut of forager worker bees to correlate to the sites of action, but also to investigate the possibility to use such enzymes as biomarkers of behavioral and biochemical changes in insects. In the present study, the analysis of data resulting from control and pesticidal administration for 24 h onto foragers allowed excellent comparisons to be made between these toxic pesticides in their formulated form. The results showed differences in the reaction of honey bees to the tested pesticides, probably due to the different degree and mode of action of each pesticide. The present study revealed that all doses of tested pesticides induced increase in GST activity that was significant at the conc-2. Metabolic pesticide resistance in insects is mediated by three major groups of detoxifying enzymes: the cytochrome P450 monooxygenases (P450s), the carboxylesterases, and GST. GST is a detoxifying enzyme that catalyzes the conjugation of a variety of electrophillic substrates to the thiol group of glutathion (GSH), producing less toxic forms, and it appears to contribute to cellular protection against oxidative damage (Hayes *et al*, 2005; Mohamed *et al*, 2015). Increased levels of GST have been associated with higher resistance to a wide variety of insecticides. In the present study, the increase in GST activity, reaching up to 186.71% of control activity strongly suggests the induction of oxidative stress by Dimethoate. On the other hand, dinotefuran inhibited GST at all tested recommended doses. Induction of GST activity has been reported in many insects following treatment with insecticides such as dithiocarb, cypermethrin, dimethoate, and chlorpyrifos (Singh *et al*, 2006). Badiou-Bénéteau *et al* (2012) added that thiamethoxam caused 119 and 156% increase in both GST and catalase activities, respectively. This induction may be due to the glutathione dependent enzymes system that provides major protection against the toxic agents.

CONCLUSION

The present study evaluated the toxicity of Dimethoate, Malathion, Quinalphos and biopestcide Neem oil on foraging workerbees of *A. mellifera* L. by using two sublethal concentrations of the pesticides for treatment. The activity of GST enzyme was determined to explore the opportunity of using this enzyme as biomarkers for honey bees exposure to pesticides. The results indicated that Dimethoate, an organophosphate was most toxic to bees among the used pesticides especially at conc-2 followed by Malathion and Quinalphos. It is therefore, suggested that this pesticide must be used with great care to avoid its drastic negative effects on non target insects such as honeybees. This is also to be noticed that neem oil, a biopesticide was not found as toxic as rest three organophosphates and it may be due to its totally different chemical structure and mode of action from organophosphates. The biopesticide Neem oil caused no significant change in GST activity. Thus, our results are of great importance as they can be used as guidelines regarding which pesticides may be toxic to these insects. Our results indicated that GST activity rapidly increased after exposure to these pesticides at all tested concentrations and its specific activity depended on the dose–response curves. Therefore, the sublethal concentrations of pesticides can be useful in monitoring the environmental toxicity of these pesticides on honey bees. However, in some cases, the use of some biomarkers as an indicator of sublethal pesticide exposure is not feasible, at least not using easy-to-handle methods in the field.

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