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Induction of Insecticide Detoxifying Enzymes in *Plutella xylostella* (L.)

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The enzyme activity in the field population of diamondback moth was assessed and mixed function oxidase (MFO) was high in all the three locations compared to glutathione-S-transferase (GST) and carboxyl ester hydrolase (CEH). Results revealed that there was high increase in MFO enzyme activity in Coimbatore, Oddanchatram and Udhagamandalam populations due to exposure of insecticides. MFO activity was high in Oddanchatram population recording 98.72, 82.29 and 96.00 per cent as influenced by the application of fenvalerate, carbosulfan and monocrotophos, respectively after 12 h of treatment than Coimbatore and Udhagamandalam populations. The specific activity of GST varied from 367.34 to 506.29, 370.26 to 512.26 and 370.26 to 455.56 at 12 h after the application of fenvalerate, carbosulfan and monocrotophos, respectively in all the three populations. CEH activity was noticed only in the case of fenvalerate and it ranged from 1554.23 to 2379.12, 1355.82 to 2186.21 and 1554.23 to 2025.46 at 6 h after application in Coimbatore, Oddanchatram and Udhagamandalam, respectively.

Key words: Detoxifying enzymes, MFO, GST, CEH, Resistance, Insecticide, *Plutella xylostella*.

Insect metabolism plays a vital role in the development of insect resistance to insecticide, such as organophosphates, carbamates and synthetic pyrethroids. The detoxifying enzymes involved in the metabolic resistance are Mixed Function Oxidase (MFOs), Glutathione-S-Transferase (GST), Carboxyl Ester Hydrolase (CEH) and acetylcholine esterases. These enzymes, which occur at low levels in susceptible strains and at high levels in resistant strains, were inducible. A chemical or substrate can induce more than one enzyme as in the case of juvenile hormone analog which induced DDT dihydrochlorinases as well as MFOs (Terriere and Yu, 1973). Diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera; Plutellidae) possesses versatile and multiple resistance mechanism to overcome toxicity of insecticides. The faster degradation of insecticides by metabolic enzymes was one such mechanism commonly associated with insecticide resistance. The involvement of glutathione-S-transferase, carboxylesterase and microsomal monooxygenase in insecticide resistance has been reported in many insect species (Brown and Brogdon, 1987; Devonshire and Field, 1991; Sun, 1992; Yu and Nguyen, 1992).

MFOs involved in insecticide metabolism were associated with microsomal fraction of tissue homogenate, derived from the endoplasmic reticulum of the intact cell. They exhibit an unusual degree of non- specificity for fat soluble compounds, which they metabolize through hydroxylation, dealkylation, oxidation, epoxidation and desulfuration (Nagatsugawa and Morelli, 1976). Cheng *et al*., (1986) reported that DBM resistance to synthetic pyrethroids, carbofuran and carbaryl, propoxur and methomyl were due to three separate MFOs. GSTs, a family of proteins have been demonstrated to be involved in detoxification of endogenous and xenobiotic compounds in vertebrates and invertebrates. Cheng *et al*. (1984) recorded two to four times more activity of GST in the DBM strain resistant to O-ethyl and O-methyl groups than in the susceptible strain. Increased activity of GST was also noticed with mevinphos resistant strain of DBM by Cheng *et al*. (1984). Some GSTs catalyze a dehydrochlorination reaction using reduced glutathione as a cofactor rather than a conjugate (Clark *et al*., 1984). These enzymes play a major role in the metabolism of organophosphates in which either the alkyl or aryl substituents on phosphorus can be cleaved (Dauterman, 1985). These enzymes exist in multiple molecular forms with distinct physical and catalytic properties. Purification by affinity chromatography and preparative isoelectric focusing revealed two groups of enzymes, the first one having relatively low isoelectric focusing points and high specificity for chlorodinitrobenzene over dichloronitrobenzene as substrate and the second one having relatively high isoelectric points and less substrate specificity (Clark *et al*., 1984 and 1986).

Insect esterases perform both physiological and defensive functions and are found in both soluble and membrane-bound forms. One of the most vulnerable sites in malathion molecule for metabolic degradation by CEH is the ethoxy carbonyl moiety. Resistance to malathion in mosquito (Matsumura and Brown, 1961), spider mite (Matsumura and Voss, 1965) and house flies (Welling and Blaakmeer, 1971) was

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attributed to CEH. Esterases were reported to be the factors responsible for the resistance in DBM to methomyl (Feng and Sun, 1978), permethrin (Liu *et al*., 1984) and fenvalerate (Cheng *et al*., 1988). Carboxyl-esterases were reported to be one of the major enzyme systems for malathion detoxification in DBM (Sun *et al* ., 1986). On the other hand, fenvalerate-resistant DBM would become susceptible to fenvalerate when the selection pressure was released and the strain would regain its resistance to fenvalerate in one generation once malathion was used to screen the DBM. Yet, Yu and Nguyen (1992) and Yu (1993) found that monoxygenases of the DBM are the major enzyme system for degrading malathion and fenvalerate. Taking into consideration the above facts, investigations were carried out to study the induction of insecticide detoxifying enzymes when insecticides were applied on the larvae.

Materials and Methods

The enzyme assays were done, to assess their involvement in insecticide resistance build-up as well as to study the induction of enzymes when insecticides were applied on larvae. The larvae were collected from different locations of Western Tamil Nadu *viz*., Coimbatore, Oddanchatram and Udhagamandalam and it was continuously reared in the laboratory under pesticide free condition. Assays were done for the estimation of mixed function oxidases, glutathione-S-transferase and carboxyl ester hydrolase in the larvae from different locations . The specific activity (SA) of each enzyme was assayed and expressed in n moles min^{-1} mg of $protein^{-1}$ and the protein estimation was done by following the method of Bradford (1976). Residue bioassay was carried out using glass vial of 20ml capacity. One ml of insecticide (fenvalerate (115 ppm), monocrotophos (140 ppm), and carbosulfan (4 ppm)) was prepared in acetone and pipetted into each of the vials, rolled to obtain an even coating of the insecticide to the inner surface of the vials and dried for one hr. Third instar larvae were released into the vials, their mouths were covered with muslin cloth and secured with rubber bands. For each treatment four replications with twelve larvae were maintained. Vials treated with acetone alone were kept as control. The larvae were collected after 1, 6 and 12 hours after treatment (HAT) for enzyme induction studies.

Mixed Function Oxidases (MFO) assay

MFO is assayed by following method of Cheng *et al*. (1986). Reagents required for this study were; Sucrose medium containing 0.24 M sucrose, 1 mM ethylene dinitro tetra acetic acid (EDTA), 1 per cent polyvinyl pyrrolidine (PVP), 5 mM phenyl methyl sulfonyl fluoride (PMSF).They were mixed in 2:1:2:1 ratio, Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) 0.5 mM, 50 mM Tris buffer, pH 7.8, 0.01 M p-nitroanisole (PNA), 1 M sodium hydroxide, Standard para-nitrophenol (Stock - 0.139 g of para-nitrophenol in 100 ml ethanol) and working standard (1ml of standard stock solution was made upto 10 ml using 50 mM Tris buffer of pH 7.8).

Ten larvae were homogenized in 5ml of sucrose medium for each insecticide using pre-chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 min and resultant supernatant was again centrifuged at 16,000 rpm for 30 min. The supernatant was used as enzyme source. Assays were made in the third instar larvae. To 500ml of enzyme source, 500ml of Tris buffer and 20ml of p-nitroanisole were added in a test tube. To this 50ml of NADPH was added in dark and the tubes were allowed to incubate for 30min The reaction was stopped by adding 500ml of sodium hydroxide. The reaction mixture was centrifuged at 10,000 rpm for 30 min. The absorbance of the supernatant was determined at 400nm. The specific activity (SA) of the enzyme was calculated using the formula, SA= ((mg of p-nitro phenol released/19.11) x (1/30) x (1000/mg of protein)) x1000.

Where, 139.11 is the molecular weight of p-nitrophenol and expressed as n moles of p-nitro phenol released $minute^{-1}$ mg of $protein^{-1}$.

Glutathione-S-transferase (GST) assay

Glutathione-S-transferase was assayed by following the method of Kao *et al*. (1989). Tris - buffer, 0.2M, pH 8, Glutathione (GSH), 0.2 M 1- chloro 2, 4-dinitrobenzene (CDBN) were the reagents. Ten larvae of DBM were homogenized in five ml of ice-cold 0.2M Tris buffer, using pre-chilled pestle and mortar. The homogenate was centrifuged at 10000 rpm for 10min in a refrigerated centrifuge at 4°C. The supernatant was collected and used as enzyme extract. Assays were taken up both in third and fourth instar larvae. The silicon sample cuvette was equilibrated at $37_{o}C$ and 20ml of CDNB in ethanol was transferred into it. Two ml GSH solution along with one ml enzyme extract were also added. To the blank, instead of substrate solution, 20ml of ethanol was added. The samples were incubated for five min and the change in absorbance was measured 30, 60, 90, 120 seconds at 340nm. Optical density (OD) difference for every 30 seconds was calculated. The
specific activity (SA) was calculated and expressed in n moles min^{-1} mg $protein^{-1}$.

Carboxyl Ester Hydrolase (CEH) assay

The methodology developed by Devonshire (1977) was adopted for carboxyl ester hydrolase assay. Phosphate buffer, 20 mM, pH 8.0, Substrate solution (1.862g a-naphthyl acetate solution dissolved in 100ml acetone), Coupling reagent (0.3 g Fast blue RR salt solution in 30ml distilled water and 3.5 g Sodium lauryl sulphate solution in 70ml distilled water were mixed at 2:5 ratio), 10mM a-naphthol standard stock solution (0.144 g in 100ml of acetone) and 0.1mM working standard (1ml of stock was made up to 100ml using 20mM phosphate buffer) were the key reagents used in the assay.

Ten insecticide evated larvae of DBM were homogenized in ice-cold 20mM phosphate buffer containing 0.2 per cent Triton X-100 using pre-chilled pestle and mortar. For the extraction, five ml of

buffer was used. The homogenate was centrifuged at 15,000 rpm in a refrigerated centrifuge at 4°C for 10 min. The supernatant was separated and stored at 4°C which served as enzyme source. The working substrate solution (5ml) was mixed with 1ml of enzyme extract. After 30 min of incubation at 27°C, one ml of coupling reagent was added. Due to reaction a red colour developed immediately, which later turned to fairly stable blue and it was measured at 600nm. The specific activity (SA) of the enzyme was estimated using the formula,

SA = ((mg of a-naphthol released/144) x (1/30) x (1000/ mg of protein)) x 1000

Where, 144 is the molecular weight of a-naphthol and SA was expressed as n moles of a-naphthol released per minute per mg of protein.

Results and Discussion

The specific activity of mixed function oxidases (MFOs), glutathione-S -transferase (GST) and carboxyl ester hydrolase (CEH) at different intervals in the larvae of DBM after application of insecticides viz., fenvalerate, carbosulfan and monocrotophos as presented in the Table 1-3. The activity of MFOs, GST and CEH after the application of fenvalerate is presented in Table 1. Increased MFOs enzyme activity was observed in Coimbatore, Oddanchatram and Udhagamandalam populations due to exposure of fenvalerate. However, MFO activity was more in Oddanchatram population (98.72%) at 12 h after treatment. In Coimbatore and Udhagamandalam only 67.61 and 41.67 per cent activity was noticed. There was marginal increase in GST enzyme content after 12 h exposure in all the three locations *viz*., Coimbatore (17.58%), Oddanchatram (16.48%) and Udhagamandalam (32.30%). The specific activity of CEH was maximum at 6h after treatment. The CEH activity ranged from 1554.23 to 2379.12 in Coimbatore, 1355.82 to 2186.21 in Oddanchatram and 1554.23 to 2025.46 in Udhagamandalam (Table 14). After 6 h, CEH activity increased maximum by 53.07, 61.25 and 30.32 per cent in Coimbatore, Oddanchatram and Udhagamandalam, respectively. The results of enzyme activity influenced by carbosulfan revealed that, the specific activity of MFOs ranged from 149.70 to 449.28 in Coimbatore population, 342.31 to 624.01 in Oddanchatram and 599.05 to 798.73 in Udhagamandalam. The variations were statistically significant. After 12 h the activity increased by 72.57, 82.29 and 16.67 in Coimbatore, Oddanchatram and Udhagamandalam, respectively (Table 2). The specific activity of GST varied from 370.26 to 512.26 at different intervals in all the three populations. The specific activity ranged from 370.26 to 462.19, 379.65 to 456.38 and 382.68 to 512.26 in Coimbatore, Oddanchatram and Udhagamandalam, respectively. The specific activity of CEH varied from 1133.29 to 1591.44 significantly at different intervals on all the three populations. The specific activity ranged from 1350.31 to 1591.44, 1133.29 to 1355.82 and 1374.42 to 1554.23 in Coimbatore, Oddanchatram and Udhagamandalam, respectively (Table 2). With regard to the influence of monocrotophos on the specific activity of MFOs, there was increased enzyme activity in Oddanchatram and Coimbatore by 96.00 and 89.33 per cent, respectively after 12 h. whereas in Udhagamandalam, it was 15.50 per cent. The specific activity of GST ranged from 370.26 to 402.10, 379.65 to 420.00, and 382.68 to 455.56 in Coimbatore, Oddanchatram and Udhagamandalam, respectively (Table 3). The specific activity of CEH increased significantly, immediately (after an hour) in all the three populations after the application of monocrotophos. The activity ranged from 1543.21 to 1896.86, 1355.82 to 1800.41 and

Table 1. MFOs, GST and CEH enzyme induction in *P. xylostella larvae* due to fenvalerate

HAT	MFO		GST		CEH	
	EA	POC	EA	POC	EA	POC
			Coimbatore			
1	280.40$_a$	7.70	367.34$_a$	-0.79	1600.67$_a$	2.99
6	340.12$_b$	30.64	401.23$_b$	8.36	2379.12$_c$	53.07
12	436.36$_c$	67.61	435.36$_b$	17.58	1818.78$_b$	17.02
Control	260.35$_a$	-	370.26$_a$	-	1554.23$_a$	-
			Oddanchatram			
1	382.37$_a$	11.70	376.63$_a$	-0.80	1280.54$_a$	-5.55
6	665.61$_b$	94.44	426.56$_b$	12.36	2186.21$_c$	61.25
12	680.25$_b$	98.72	442.23$_b$	16.48	1543.21$_b$	13.82
Control	342.31$_a$	-	379.65$_a$	-	1355.82$_a$	-
			Udhagamandalam			
1	611.79$_a$	-10.64	378.16$_a$	-1.18	1649.92$_a$	6.16
6	766.78$_b$	12.00	483.62$_b$	26.38	2025.46$_c$	30.32
12	969.88$_c$	41.67	506.29$_b$	32.30	1846.34$_b$	18.79
Control	684.62$_a$	-	382.68$_a$	-	1554.23$_a$	-

EA - Enzyme activity in moles min^{-1} mg protein^{-1}, HAT – Hours after treatment, Means followed by common letter in a column are not significantly different at five per cent level by DMRT. MFO – Mixed Function Oxidase, GST – Glutathione-S-transferases, CEH – Carboxyl Ester Hydrolase. POC-Percentage over Control.

Table 2. MFOs, GST and CEH enzyme induction in *P. xylostella larvae* due to carbosulfan

HAT	MFO		GST		CEH	
	EA	POC	EA	POC	EA	POC
			Coimbatore			
1	149.76$_a$	-42.48	415.36$_b$	12.18	1446.76$_b$	-6.91
6	324.48$_c$	24.63	439.64$_b$	18.74	1350.31$_a$	-13.12
12	449.28$_d$	72.57	462.19$_c$	24.83	1591.44$_c$	2.39
Control	260.35$_b$	-	370.26$_a$	-	1554.23$_c$	-
			Oddanchatram			
1	389.38$_a$	13.75	410.32$_b$	8.08	1215.28$_b$	-10.37
6	449.28$_b$	31.25	425.69$_b$	12.13	1133.29$_a$	-16.41
12	624.01$_c$	82.29	456.38$_c$	20.21	1229.75$_b$	-9.30
Control	342.31$_a$	-	379.65$_a$	-	1355.82$_c$	-
			Udhagamandalam			
1	629.00$_b$	-8.13	446.37$_b$	16.64	1417.82$_b$	-8.78
6	599.05$_a$	-12.50	493.16$_c$	28.87	1374.42$_a$	-11.57
12	798.73$_c$	16.67	512.26$_c$	33.86	1543.21$_c$	-0.71
Control	684.62$_b$	-	382.68$_a$	-	1554.23$_c$	-

EA - Enzyme activity in moles min^{-1} mg protein^{-1}, HAT – Hours after treatment, Means followed by common letter in a column are not significantly different at five per cent level by DMRT. MFO – Mixed Function Oxidase, GST – Glutathione-S-transferases, CEH – Carboxyl Ester Hydrolase. POC-Percentage over Control.

Table 3. MFOs, GST and CEH enzyme induction in *P. xylostella larvae* due to carbosulfan

HAT	MFO		GST		CEH	
	EA	POC	EA	POC	EA	POC
			Coimbatore			
1	432.64$_b$	66.18	376.49$_a$	1.68	1896.86$_c$	22.04
6	505.86$_c$	86.62	398.79$_a$	7.71	1543.21$_a$	-0.71
12	670.93$_d$	89.33	402.16$_a$	8.62	1620.37$_b$	4.26
Control	260.35$_a$	-	370.26$_a$	-	1554.23$_a$	-
			Oddanchatram			
1	399.36$_a$	16.67	388.73$_a$	2.39	1800.41$_c$	32.79
6	505.86$_b$	47.78	410.26$_a$	8.06	1594.65$_b$	17.62
12	670.93$_c$	96.00	420.60$_a$	10.79	1643.52$_b$	21.22
Control	342.31$_a$	-	379.65$_a$	-	1355.82$_a$	-
			Udhagamandalam			
1	765.45$_b$	11.81	405.36$_a$	5.93	1896.86$_b$	22.04
6	718.86$_a$	5.00	436.19$_a$	13.98	1568.93$_a$	0.95
12	790.74$_b$	15.50	455.56$_b$	19.04	1550.93$_a$	-0.21
Control	684.62$_a$	-	382.68$_a$	-	1554.23$_a$	-

EA - Enzyme activity in moles min^{-1} mg protein^{-1}, HAT – Hours after treatment, Means followed by common letter in a column are not significantly different at five per cent level by DMRT. MFO – Mixed Function Oxidase, GST – Glutathione-S-transferases, CEH – Carboxyl Ester Hydrolase. POC- Percentage over Control.

1550.93 to 1896.86 in Coimbatore, Oddanchatram and Udhagamandalam, respectively. The highest increase in the CEH enzyme activity was noticed after 1 h insecticide treatment (22.04, 32.79, and 22.04 per cent, at Coimbatore, Oddanchatram and Udhagamandalam, respectively) and later the activity decreased drastically.

The DBM possesses a versatile and multiple resistance mechanisms to overcome toxicity of insecticides. The faster degradation of insecticides by metabolic enzymes is one such major mechanism commonly associated with insecticide resistance. The involvement of mixed function oxidase (MFOs), glutathione-S-transferase (GST) and carboxyl ester hydrolase (CEH) in insecticide resistance has been reported in insecticide resistance strains of many insect species (Brown and Brogdon, 1987; Devonshire and Field, 1991; Sun, 1992; Yu and Nguyen, 1992; Duraimurugan, 2004). Higher activity of MFOs, GST and CEH has been observed in the resistant field population collected at different locations in the present investigation similar to earlier reports (Bhat, 1999). Among the detoxifying enzymes studied MFO exhibited substantially higher activity which was evidenced from the induction enzyme in the larvae treated with insecticides. Among the three populations, Oddanchatram exhibited maximum of 98.72, 82.29 and 96.00 per cent activities over control with fenvalerate, carbosulfan and monocrotophos, respectively 12 h after application, which was followed by Coimbatore (67.61, 72.57 and 89.33%) and Udhagamandalam population recorded low level of 41.67, 16.67 and 15.50 per cent for fenvalerate, carbosulfan and monocrotophos, respectively. The present study clearly indicated that MFO played a dominant role in the oxidation of fenvalerate, carbosulfan and monocrotophos. This in line with earlier workers who have reported the involvement of this enzyme in the detoxifying metabolism of organophosphates and synthetic pyrethroids to DBM (Yu and Nguyen, 1996; Bhat, 1999; Singh, 2002; Mohan and Gujar, 2003).

There is a marginal increase in GST activity (8.62 to 33.86%) 12 h after the application of fenvalerate, carbosulfan and monocrotophos. The increased activity of GST in the resistance population of DBM in the present study, confirm the involvement of this enzyme in insecticide resistance.GST degrade the insecticides by their ability to dealkylate, dearylate, dehalogenate or conjugate, depending up on the insecticide involved in resistance development in the test insect. The activity of GST found in the field population in the present study was also reported by Balabaskaran *et al*. (1989) who found 3-4 fold increase in activity in resistant strain. Unlike the other enzymes, the CEH was not found with carbosulfan and monocrotophos but the specific activity of CEH, 6 h after application of fenvalerate showed an increase of 53.07, 61.25 and 30.32 in Coimbatore, Oddanchatram and Udhagamandalam, respectively. Among the pyrethroid, high level of CEH activity in *Helicoverpa armigera* to fenvalerate was reported by Ramasubramanian (2003)

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