Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2017; 5(5): 1755-1763

Received: 12-07-2017
Accepted: 13-08-2017

Ram K Wargantiwar
Department of Entomology, Punjab Agricultural University, Ludhiana, India

BK Kang
Department of Entomology, Punjab Agricultural University, Ludhiana, India

Correspondence
Ram K Wargantiwar
Department of Entomology, Punjab Agricultural University, Ludhiana, India

Basis of biochemical mechanisms to fenvalerate resistance populations of tomato fruit borer from Punjab, India

Ram K Wargantiwar and BK Kang

Abstract
Different populations of *H. armigera,* were collected from major tomato growing districts (Amritsar, ASR; Kapurthala, KPT; Patiala, PTA) of Punjab were treated in the laboratory by leaf dip method with fenvalerate alone and in combination with different ratios of synergists like Piperonyl butoxide (PBO), Tri phenyl phosphate (TPP) and Diethyl maleate (DEM) at ratios of 1:2, 1:3 and 1:5. The biochemical estimations were done with resistant populations collected from Amritsar (ASR) along with the susceptible population procured from National Bureau of Agricultural Insect Resources, Bengaluru (NBAIR) and fenvalerate selected strain (ASR-Sel strain) developed in the laboratory by giving selection pressure with fenvalerate up to 10 generations. The result obtained in the present study revealed that PBO at 1:5 had highest synergistic effect on the selected resistant ASR-Sel (9.13-fold) and resistant ASR (7.06-fold) strains as compared to the susceptible population (2.50-fold). However, TPP and DEM showed slight synergism with the combination of fenvalerate in all the tested ratios. A strong positive correlation (r= 0.99) between monooxygenases and pyrethroid resistance in *H. armigera* with MFO activity 4.02 and 2.05 fold in ASR-Sel and ASR population was observed as compared to susceptible one. The present studies were conducted to investigate the various biochemical mechanisms involved in imparting resistance in tomato fruit borer, *H. armigera* during 2014-15 at Toxicology Laboratory, Department of Entomology and Department of Biochemistry at Punjab Agricultural University, Ludhiana.

Keywords: *Helicoverpa armigera*, fenvalerate, synergists, monooxygenases, esterase, acetylcholinesterase, glutathione-S-transferases

1. Introduction
India is the second largest tomato producer in the world after China, accounting for about 11.5 per cent of the world tomato production during 2014-15, the area and production of tomato, in India, was about 893000 ha and 19166000.7 MT, respectively [3]. The tomato fruit borer, *Helicoverpa armigera* (Hübner) is one of the most destructive pests of field crops causing economic losses up to 54.82 per cent [26]. The *H. armigera* has been recorded feeding on 182 plant species across 47 families in the Indian subcontinent, of which 56 are heavily damaged and 126 are rarely affected [39]. Tomato production is hampered by attack of different insect pests such as tomato fruit borer, leaf miner, thrips, mealy bug, red spider mites etc. Among these tomato fruit borer, *H. armigera* is one of the most destructive polyphagous pest which causes annual loss of over $2 billion in the semiarid tropics, despite the applications of various insecticides costing $500 million annually [44]. The management of *Helicoverpa* has become increasingly difficult due to indiscriminate and extensive use of chemical insecticides in past few decades which has led to development of resistance to most commonly used chemical class of insecticides [6, 28, 41]. Insecticide resistance in *H. armigera* in India was first recorded in 1987, when farmers in the coastal districts of Andhra Pradesh were unable to control the very high populations of *H. armigera* on their cotton crops with conventional insecticides [14]. Similarly, a high level of resistance to various insecticides including organochlorines, organophosphates and pyrethroids has been reported from various parts of the world, including India [6, 20, 24, 31, 34, 36]. Even within new chemistries the pest has been reported to have developed resistance [30, 48]. The development of resistance may be delayed but the possibility for development of resistance cannot be totally denied.
Synergists increase the lethality of insecticides by inhibiting insecticide detoxifying enzymes. This enables synergists the tools for elucidating resistance mechanisms, especially if they are

specific inhibitors of a particular resistance conferring mechanism such as detoxification of enzymes and also plays a significant role in enhancing toxicity on the resistant strain to a greater extent.

Keeping in view the losses, failure of management and development of resistance to recommended insecticides in Punjab, India against *H. armigera*, the present investigation was designed to identify the relative importance of oxidases and esterases contributing to pyrethroid resistance in a resistant strain of *H. armigera* selected with a fenvalerate mixture with combined evidences from synergist bioassay and metabolic enzyme analysis.

2. Material and Methods

The present studies were conducted to investigate the various biochemical mechanisms involved in imparting resistance in tomato fruit borer, *H. armigera* during 2014-15 at Toxicology Laboratory, Department of Entomology and Department of Biochemistry at Punjab Agricultural University, Ludhiana.

2.1 Collection and rearing of insects

The populations comprising of egg masses and larvae of *H. armigera* were collected from infested plants in various tomato growing areas of Punjab *viz*; Amritsar (31°37 ′N, 74°51′ E), Kapurthala (31° 22' 45" N, 75° 23' 5" E) and Patiala (30°19′ N, 76°24′ E) during the period from March to April 2013 and 2014 and brought to the laboratory in plastic jars covered with muslin. Each larva was kept in separate vials along with food. All the larvae were reared in specimen tubes singly and the culture was maintained in B.O.D. incubator maintained at 25 ± 1ºC and 65 ± 5 per cent relative humidity. A semi-synthetic diet as per Armes was used for larval rearing [5]

The larvae were first reared in group of 100-200 in wide plastic vials containing 2-5 mm thick layer of artificial diet. After 4-5 days, when the larvae become cannibalistic in nature, they were transferred to 50 ml cups separately containing artificial diet and were allowed to feed until the pupation. After emergence ten pairs of moths were released in glass jars (10 x 15 cm) for further rearing. Before releasing into the jars, ensured that the jars were covered by black papers to maintain darkness as well as the upper orifice was closed with muslin. Cotton swab dipped in 10% honey solution was provided as a food for the moths. Muslin of each jar was examined daily, if the egg laying is found was removed and placed in a separate jar.

The population initially collected from Amritsar (ASR) was found to be most resistant to fenvalerate among the three populations of *H. armigera.* The susceptible population (SUS) procured from the National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India was reared on chick pea based semi synthetic diet in insecticidal free environment. The insecticide resistant (ASR), susceptible (SUS) and insecticide resistant selected strains (ASR-Sel) of *H. armigera* were used to assess the various biochemical mechanisms involved in imparting resistance through synergism and enzyme assays.

2.2 Insecticides and Chemicals

Commercial formulations of insecticide were used for bioassay in synergism comprised fenvalerate 25 EC (Sumicidin, New Chemical Industries Ltd, Gujarat). The toxicity of fenvalerate was also evaluated in the presence of three synergists, Piperonil butoxide (3, 4- methylenedioxy- 6 - p r o p y l benzy l - n-butyl diethyleneglycolether) or PBO from Sigma Aldrich Co., an inhibitor of cytochrome P_{450} monooxygenases and of esterases, Diethyl maleate (DEM) from Himedia Laboratories Pvt Ltd, and Tri phenyl phosphate (TPP) from Loba Chemie Lab Reagents and Fine Chemicals, an inhibitor of GST and esterases. Mono sodium dihydrogen phosphate anhydrous, Di sodium dihydrogen phosphate anhydrous, Editic acid (EDTA), Triton X-100, Nicotinamide adenine dinucleotide phosphate reduced tetra sodium salt (NADPH), 5,5'- dithiobis dinitro benzene Ellman's Reagent (DTNB), α-naphthol, Acetone, Sodium hydroxide, Copper sulphate, Sodium potassium tartarate were from S.d. finechem Ltd, Mumbai. 1-phenyl, 2-thiourea (PTU), *p*-nitro anisole, Fast Blue RR, Bovine Serum Albumin was from Sigma Aldrich Co. Acetyl thiocholine iodide (ATChI) was supplied by Himedia Labs Pvt Ltd, Mumbai.

2.3 Establishment of fenvalerate selected resistant strain

H. armigera population collected from Amritsar (ASR) that was found to be the most resistant was selected with fenvalerate for ten generations. The progeny of field-collected ASR population was exposed to a series of concentrations and LC_{50} was determined. The individuals surviving from the treated population at concentrations LC_{50} were collected and reared to obtain a batch of first selected generation that was chosen as F_1. The larvae from F_1 generation were also exposed to single selective concentration equivalent to LC_{50} va1ue. The third instar larvae (30-50 mg) of F_2 were treated-with different concentrations to calculate LC_{50} for that generation and individuals surviving at LC_{50} were reared further. This LC_{50} value generated was used to select two subsequent generations. Similar procedure was followed for the F_5, F_8 and F_9 generations where LC_{50} values were worked out and subsequently used for selection of two succeeding generations. The number of larvae subjected to selection in each generation varied depending upon the number and vigor of the previous generation. Finally, the LC_{50} values were calculated for the F_{10} generation, chosen as ASR-Sel.

2.4 Bioassay and synergism studies

H. armigera populations identified as susceptible (SUS), resistant (ASR) and fenvalerate selected population (ASR-Sel) were tested to assess the degree of PBO, DEM and TPP mediated resistance acquired. The insecticide was tested alone and in combination with synergists (1:2, 1:3 and 1:5). The dilutions required were prepared from commercial formulations. Different concentrations of these synergists were prepared in distilled water by serial dilution technique. Following a preliminary dosing, the concentrations to be used were finalized for each synergist *viz*., PBO, TPP, DEM at 1:2 (0.75-20.0 µg/µl), PBO, TPP, DEM at 1:3 (0.375-20.0 µg/µl) and PBO, TPP, DEM at 1:5 (0.15-20.0 µg/µl) and used distilled water as control against different selected populations to obtain a mortality ranging from 10-90 per cent. The 3rd instar larvae were treated using leaf disc dip bioassays recommended by IRAC [2]. There were four replications with ten larvae each. The mortality data was recorded after 48 h of treatment. A larva was considered dead if it failed to move in coordinated manner, when probed with a blunt needle.

2.5 Assays of protein contents

Total protein content of the enzyme solution was determined by the Lowry method using bovine serum albumin as the standard [32].

2.6 Mixed function oxidase (MFO)
MFO activity / *p*-nitroanisole o-demethylase (ODM) was assayed according to the method of Kranthi [27]. Change in absorbance was recorded at 405 nm at 15 sec interval for 20 min. The activity of MFO was recorded at 405 nm at 15 sec interval for 20 min.

2.7 Carboxyl esterase (CarE)
CarE activity was measured using α-naphthyl acetate as substrate based on the method described by Aspersen (1962).

2.8 Acetyl choline esterase (AChE)
Acetyl choline esterase (AChE) activity was examined by the method of Elliman [15].

2.9 Glutathione-S- transferases
Extraction and estimation of glutathione S- transferase was done using methodology given by Chien and Dauterman [12]. The assay mixture consisted of 30 µL of ethanolic CDNB solution, 100 µL GSH solution and 50 µL of crude enzyme solution with 0.1 M sodium phosphate buffer (pH 7.6) containing PTU in a total volume of 2 ml. Absorbance was recorded at 340 nm at intervals of 1 min for 5 min. The activity of GST was expressed in terms of µM of CDNB conjugated min^{-1} mg^{-1} protein.

2.4 Statistical analysis
The mortality data was subjected to Abbott's correction before computing LC_{50} values by Probit Analysis [1, 17]. Synergistic factor was calculated by comparing the LC_{50} values of an insecticide alone with that of LC_{50} value of an insecticide + PBO/ TPP/ DEM mixture. Data were expressed as Mean ± standard error. Data were statistically analyzed by (ANOVA) test using CPCS-1, and the differences were considered statistically significant at $p < 0.05$.

3. Results
3.1 Toxicity of fenvalerate to resistant (ASR) and susceptible (SUS) populations of *H. armigera*
The susceptible population (SUS) was procured from the National Bureau of Agricultural Insect Resources (NBAIR), Bengaluru, India and was reared on chickpea based semi synthetic diet in insecticidal free environment. The *H. armigera* population collected from Amritsar (ASR) that was found to be the most resistant and selected as a resistant population. The dose mortality relationship worked out for Amritsar (ASR) resistant and susceptible (SUS) strain is presented in Table 1 with Log LC_{50} values 1.27 and 0.05 (µg/µl) against resistant and susceptible strains, respectively.

Table 1: Toxicity of fenvalerate to the susceptible (SUS) and resistant (ASR) strains of *H. armigera*

Locations	LC_{50} µg/µl	LC_{90} µg/µl	Slope	FL at 95% CL	Heterogeneity χ^2 (df)
SUS	0.05	0.23	1.914 ± 0.195	0.003- 0.006	1.23 (7)
ASR	1.27	3.06	3.362-0.440	0.105-0.154	0.74 (5)

3.2 Selection of field population with fenvalerate
The selection process of the fenvalerate resistant strain is shown in Table 2. The LC_{50} of fenvalerate in the initial population ASR was (1.27µg/µl) (highest among the populations). For the resistant strain, the LC_{50} value of population was (2.47 µg/µl) after six^{th} generation of selection and the resistance level of the third instar larvae to fenvalerate increased 1.95 times than other. Selection pressure up to 10 generations resulted in an increase in the LC_{50} of fenvalerate to 5.39 (µg/µl) in the selected strain (ASR-Sel). This indicates 4.25 fold increases in the resistance level. The 95 per cent confidence limit did not overlap in two populations, hence these can be considered as significantly different. There was increase in slope value from 3.362 to 5.541, which indicates a considerable heterogeneity in the response of strain, suggesting a greater potential for the development of higher resistance (Table 2, Fig. 1)

Table 2: Selection of resistance to fenvalerate in *H. armigera*

Locations	LC_{50} (µg/µl)	LC_{90} (µg/µl)	Slope	FL at 95% CL	Heterogeneity χ^2 (df)	RR*
F_0 ASR	1.27	3.06	3.362 ± 0.440	0.105- 0.154	0.74 (5)	1.00
F_2	1.58	3.82	3.346 ± 0.426	0.120- 0.208	1.15 (5)	1.24
F_4	1.93	4.48	3.449 ± 0.448	0.139- 0.271	1.63 (5)	1.51
F_6	2.47	5.28	3.888 ± 0.526	0.207- 0.295	0.73 (5)	1.95
F_8	3.41	7.03	4.072 ± 0.559	0.287- 0.405	0.43 (5)	2.69
F_{10}	5.39	9.17	5.541 ± 0.871	0.464- 0.628	0.57 (5)	4.25

*Resistance ratio = LC_{50} of the resistant strain/ LC_{50} of the F_0 Population

3.3 Mechanism of resistance through synergism studies
When fenvalerate mixed with different synergists PBO, DEM and TPP at ratio of 1:2 in the susceptible, resistant and selected resistant strains, the synergistic factor were found to be 1.25, 1 and 1.25 for susceptible strain (SUS) and 2.15, 1.25 and 1.46 for resistant strain (ASR) and 3.02, 1.10 and 1.38 for selected resistance (ASR-Sel) (Table 3). The results obtained in the present study revealed that PBO had highest synergistic effect on the selected strain (3.02-fold) followed by TPP (1.38-fold) and DEM 1.10-fold. In the ASR and ASR-Sel, PBO showed 2.15 and 3.02- fold synergism, respectively. PBO had stronger synergism for fenvalerate than the other two synergists. In the ASR and ASR-Sel populations DEM and TPP showed slightly synergism (1.25, 1.10 for DEM and 1.46, 1.38- fold) for TPP respectively, which suggests the involvement of esterase in imparting resistance to various insecticides like synthetic pyrethroids.

When PBO, DEM and TPP were used as a synergist at ratio 1:3 in the susceptible, resistant and selected resistant strains, the synergistic ratio was found to be 1.67, 1.25 and 1.25 for susceptible strain (SUS) and 6.35, 1.28 and 1.86 for resistant strain (ASR) and 8.42, 1.54 and 1.92 for selected resistance (ASR-Sel) (Table 4). When these synergists were used at ratio 1:5, the synergistic ratio was 2.50, 1.25 and 1.25 for susceptible strain (SUS) and 7.06, 1.30 and 2.12 for resistant strain (ASR) and 9.13, 1.69 and 2.20 for selected resistance (ASR-Sel) (Table 5).

The use of fenvalerate in mixtures with PBO at ratio of 1:2, 1:3 and 1:5 showed synergistic factor of 1.25, 1.67 and 2.50 in the susceptible lab populations, respectively whereas fenvalerate + TPP applied showed synergistic factor of 1.25 at different ratios. DEM synergized fenvalerate in various ratios (1:2, 1:3 and 1:5) and evaluated LC_{50} values as 0.05, 0.04 and 0.04 µg/µl. The synergistic factor of the fenvalerate + DEM was formed to be 1.00, 1.25 and 1.25 at the 1:2, 1:3 and 1:5, respectively (Table 3 -5).

When resistant Amritsar (ASR) population was subjected to fenvalerate mixed with PBO showed the synergistic factor of 2.15, 6.35 and 7.06 at 1:2, 1:3 and 1:5, respectively. PBO at 1:5 ratio had stronger synergism and was significantly more toxic than fenvalerate + PBO at 1:2 and 1:3. The combinations of fenvalerate with PBO produced significant synergistic effects at 1:2, 1:3 and 1:5 with synergistic value all greater than one. The same combination when offered fenvalerate with TPP showed synergistic factors of 1.46, 1.86 and 2.12 at 1:2, 1:3 and 1:5, respectively (Table 3-5). At the ratios of 1:2, 1:3 and 1:5 the DEM was slightly synergized with the combination of fenvalerate and the synergistic factor was 1.25, 1.28 and 1.30, respectively. PBO had stronger synergism for fenvalerate than other two synergists such as TPP and DEM, while it implied that oxidative metabolism could be involved in fenvalerate resistance in the resistant (ASR) *H. armigera* populations and the esterase and glutathione S-transferase mechanism of detoxification might be less important in conferring fenvalerate resistance against ASR population.

Table 3: Synergism of fenvalerate by PBO, DEM and TPP in different populations of *H. armigera* (1:2)

Insecticides	LC_{50} (µg/µl)	Fiducial limits	LC_{90} (µg/µl)	Fiducial limit	Slope ± S.E.	Heterogeneity χ^2	df	SR
SUS								
Fenvalerate	0.05	0.004-0.006	0.35	0.022-0.064	1.480 ± 0.150	0.84	7	1.00
Fenvalerate + PBO (1:2)	0.04	0.003-0.006	0.25	0.017-0.042	1.697 ± 0.169	0.37	7	1.25
Fenvalerate + DEM (1:2)	0.05	0.004-0.006	0.29	0.019-0.051	1.604 ± 0.161	0.78	7	1.00
Fenvalerate + TPP (1:2)	0.04	0.003-0.006	0.28	0.019-0.049	1.602 ± 0.161	0.63	7	1.25
ASR								
Fenvalerate	1.27	0.105-0.154	3.06	0.239-0.446	3.362 ± 0.440	0.74	5	1.00
Fenvalerate + PBO (1:2)	0.59	0.034-0.087	3.98	0.255-0.789	1.545 ± 0.239	0.40	5	2.15
Fenvalerate + DEM (1:2)	1.01	0.076-0.136	6.93	0.445-1.390	1.532 ± 0.167	0.84	6	1.25
Fenvalerate + TPP (1:2)	0.87	0.070-0.108	2.77	0.205-0.426	2.551 ± 0.296	0.79	5	1.46
ASR-Sel								
Fenvalerate	5.39	0.464-0.628	9.17	0.762-1.240	5.541 ± 0.871	0.57	5	1.00
Fenvalerate + PBO (1:2)	1.78	0.132-0.239	12.77	0.819-2.420	1.496 ± 0.165	0.50	6	3.02
Fenvalerate + DEM (1:2)	4.88	0.389-0.611	17.90	1.262-2.604	2.349 ± 0.252	0.40	7	1.10
Fenvalerate + TPP (1:2)	3.90	0.310-0.487	13.65	1.00- 2.085	2.347 ± 0.252	0.72	6	1.38

SR- Synergistic ratio = LC_{50} insecticide alone/ LC_{50} of insecticide + synergist

Table 4: Synergism of fenvalerate by PBO, DEM and TPP in different populations of *H. armigera* (1:3)

Insecticides	LC_{50} (µg/µl)	Fiducial limits	LC_{90} (µg/µl)	Fiducial limit	Slope ± S.E.	Heterogeneity χ^2	df	SR
SUS								
Fenvalerate	0.05	0.004-0.006	0.35	0.022- 0.064	1.480 ± 0.150	0.84	7	1.00
Fenvalerate + PBO (1:3)	0.03	0.002-0.004	0.10	0.007-0.014	2.809 ± 0.322	0.52	7	1.67
Fenvalerate + DEM (1:3)	0.04	0.003- 0.004	0.15	0.011- 0.023	2.181 ± 0.227	0.51	7	1.25
Fenvalerate + TPP (1:3)	0.04	0.002- 0.004	0.16	0.011- 0.024	2.050 ± 0.210	0.86	7	1.25
ASR								
Fenvalerate	1.27	0.105- 0.154	3.06	0.239-0.446	3.362 ± 0.440	0.74	5	1.00
Fenvalerate + PBO (1:3)	0.20	0.015- 0.027	1.03	0.073- 0.173	1.845 ± 0.243	0.18	5	6.35
Fenvalerate + DEM (1:3)	0.99	0.081- 1.22	2.78	0.212- 0.414	2.862 ± 0.345	0.36	5	1.28
Fenvalerate + TPP (1:3)	0.68	0.054- 0.084	2.32	0.170- 0.358	2.400 ± 0.274	0.56	5	1.86
ASR-Sel								
Fenvalerate	5.39	0.464- 0.628	9.17	0.762- 1.240	5.541 ± 0.871	0.57	5	1.00
Fenvalerate + PBO (1:3)	0.64	0.046- 0.081	2.40	0.176- 0.387	2.219 ± 0.324	0.90	5	8.42
Fenvalerate + DEM (1:3)	3.50	0.282- 0.435	10.93	0.815- 1.665	2.593 ± 0.300	0.39	5	1.54
Fenvalerate + TPP (1:3)	2.80	0.229- 0.344	7.78	0.597- 1.138	2.889 ± 0.339	0.08	5	1.92

SR- Synergistic ratio = LC_{50} insecticide alone/ LC_{50} of insecticide + synergist

Table 5: Synergism of fenvalerate by PBO, DEM and TPP in different populations of *H. armigera* (1:5)

Insecticides	LC_{50} (µg/µl)	Fiducial limits	LC_{90} (µg/µl)	Fiducial limit	Slope ± S.E.	Heterogeneity χ^2	df	SR
SUS								
Fenvalerate	0.05	0.004-0.006	0.35	0.022- 0.064	1.480 ± 0.150	0.84	7	1.00
Fenvalerate + PBO (1:5)	0.02	0.001-0.002	1.00	0.007-0.016	1.952 ± 0.202	0.46	7	2.50
Fenvalerate + DEM (1:5)	0.04	0.003-0.005	0.17	0.012- 0.025	2.187 ± 0.228	0.59	7	1.25
Fenvalerate + TPP (1:5)	0.04	0.002-0.004	0.15	0.011- 0.023	2.075 ± 0.213	0.77	7	1.25
ASR								
Fenvalerate	1.27	0.105-0.154	3.06	0.239-0.446	3.362 ± 0.440	0.74	5	1.00
Fenvalerate + PBO (1:5)	0.18	0.012-0.023	0.79	0.056- 0.133	1.971 ± 0.277	0.95	5	7.06
Fenvalerate + DEM (1:5)	0.97	0.078-0.118	2.67	0.203 – 0.396	2.891 ± 0.349	0.68	5	1.30
Fenvalerate + TPP (1:5)	0.60	0.047-0.074	2.00	0.149- 0.306	2.424 ± 0.274	0.44	5	2.12
ASR-Sel								
Fenvalerate	5.39	0.464-0.628	9.17	0.762- 1.240	5.541 ± 0.871	0.57	5	1.00
Fenvalerate + PBO (1:5)	0.59	0.040-0.778	2.59	0.185- 0.435	1.994 ± 0.297	0.60	5	9.13
Fenvalerate + DEM (1:5)	3.18	0.255-0.398	10.55	0.779- 1.630	2.460 ± 0.281	0.56	5	1.69
Fenvalerate + TPP (1:5)	2.45	0.196-0.303	7.73	0.580- 1.159	2.559 ± 0.291	0.31	5	2.20

SR- Synergistic ratio = LC_{50} insecticide alone/ LC_{50} of insecticide + synergist

3.4 Activities of detoxification enzymes

Activities of hydrolases, monooxygenases and Glutathione-S-transferase in susceptible (SUS), Amritsar resistant (ASR) and fenvalerate selected (ASR-Sel) populations are shown in (Table 6, Fig. 2). The specific activity of monooxygenases was found to be the higher in ASR-Sel (471.47 nmole min^{-1} mg^{-1}) and ASR (240.83 nmole min^{-1} mg^{-1}) compared to susceptible strain (117.17 nmoles min^{-1} mg^{-1} protein) which was significantly lower than other populations. It is likely that enhanced detoxification by MFOs is the major mechanism of pyrethroid resistance in the resistant populations. So far as esterases are concerned the populations of *H. armigera* (ASR and ASR-Sel) had significantly higher esterase activity (2.20 and 3.90 µmoles naphthol min^{-1} mg^{-1}) than in the (SUS) strain (0.98 µmoles naphthol min^{-1} mg^{-1}). Similarly the specific activity of AChE in whole body homogenate of *H. armigera* was found to be higher in the ASR-Sel (3.88 min^{-1} mg^{-1} of protein) and ASR (3.34 min^{-1} mg^{-1} of protein) as compared to the susceptible strain (1.94 min^{-1} mg^{-1} of protein). The field populations of ASR and ASR-Sel strain exhibited 2.24 4.0 and 1.72, 2.00- fold enzyme activity (Hydrolases) as compared to the susceptible strain. The activity of glutathione S- transferase in whole body homogenates of *H. armigera*, as inferred from CDNB assay, was higher in the ASR and ASR-Sel populations as compared to the susceptible strain. ASR-Sel recorded 1.70- fold enzyme activity (0.59 µmoles min^{-1} mg^{-1} of protein) and ASR had 1.29 fold GST activity (0.45 µmoles min^{-1} mg^{-1} of protein) than the susceptible strain (0.34 µmoles min^{-1} mg^{-1} of protein). Activities of different enzymes in three different populations of *H. armigera* showed a positive correlation with LC_{50} values of fenvalerate (Table 7).

Table 6: Specific activity of detoxification enzymes in different populations of *H. armigera*

Populations	Hydrolases				MFO[3]		GST[4]	
	Esterase[1] Specific activity	Ratio	AChE[2] Specific activity	Ratio	Specific activity	Ratio	Specific Activity	Ratio
SUS	0.98±0.01	1	1.94±0.12	1	117.17±7.61	1	0.34±0.011	1
ASR	2.20±0.03	2.24	3.34±0.23	1.72	240.83±9.15	2.05	0.45±0.016	1.29
ASR-Sel	3.90±0.04	4.0	3.88±0.26	2.00	471.47±11.32	4.02	0.59±0.017	1.70
LSD(p<0.05)	0.35		0.74		33.78		0.053	

Mean of three replications ± SE

SUS- Susceptible population, ASR- Amritsar resistant population, ASR-Sel Fenvalerate selected Amritsar population. MFO- Mixed function oxidase, GST- Glutathione S-transferase, AChE- Acetylcholine esterase.

[1] µmoles of α- naphthol formed min^{-1} mg^{-1} of protein.

[2] nmoles of free thiol formed min^{-1} mg^{-1} of protein.

[3] nmoles of *p*- nitrophenol formed min^{-1} mg^{-1} of protein.

[4] µmoles of 1- chloro 2, 4- dinitro benzene conjugated min^{-1} mg^{-1} of protein.

Table 7: Correlation between specific activity of enzyme and LC_{50} values of fenvalerate against *H. armigera*

Enzymes	Correlation coefficient (r)	Coefficient of determination (R^2)
Esterase	0.97*	0.978
Acetylcholinesterase	0.84	0.576
Mixed function oxidase	0.99*	0.989
Glutathione S- transferase	0.97*	0.978

r is correlation coefficient, R^2 is coefficient of determination

*Significant at $p<0.05\%$

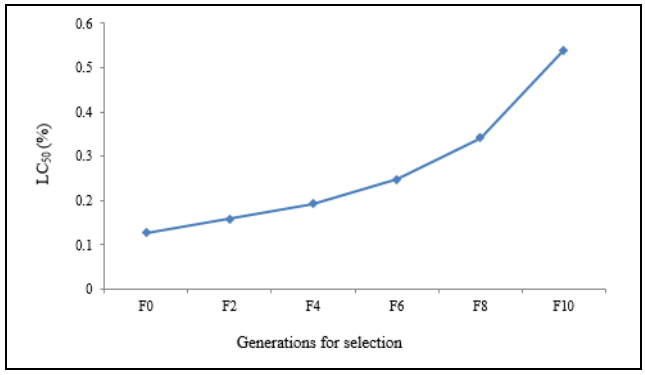

Fig 1: Development of resistance towards fenvalerate during successive selection with increasing concentrations towards the third instar over 10 generations in *H. armigera*.

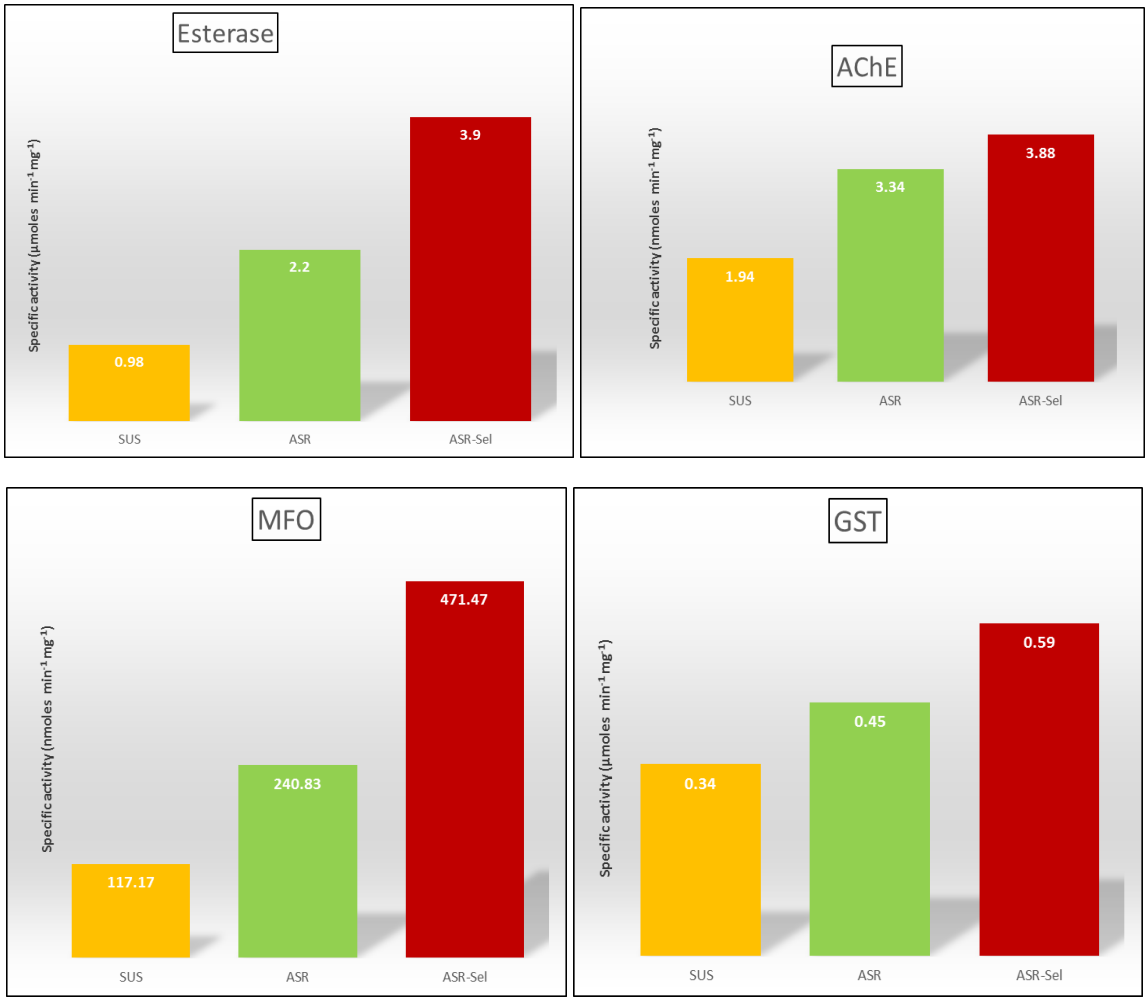

Fig 2: Specific activity of detoxification enzymes in different populations of *H. armigera* MFO- Mixed function oxidase, GST- Glutathione S-transferase, AChE- Acetylcholine esterase. SUS- Susceptible population, ASR- Amritsar resistant population, ASR-Sel fenvalerate selected Amritsar population.

4. Discussion

The result obtained in the present study revealed that the selection pressure up to 10 generations of *H. armigera* resulted in an increase in the LC_{50} of fenvalerate to 5.39 µg/µl in the selected strain (ASR-Sel) which indicates 4.25 fold increase in the resistance level. Kaur and Kang reported that selection of *S. litura* field population with pyrethroid (deltamethrin) significantly increased its resistance to 3.0 fold after 10 generations [25]. The dual insecticide + synergist mixture showed suppression of pyrethroid resistance strains by PBO for all the three locations with synergistic factor of 3.02, 8.42 and 9.13 fold at 1:2, 1:3 and 1:5 ratios, respectively (Table 3-5). The strong suppression by PBO in all the susceptible (SUS), resistant (ASR) and selected resistant (ASR-Sel) strains. These high values of synergism indicating that MFO (microsomal oxidase) inhibitors might be involved in the development of resistance in *H. armigera*. Yang reported 5.0 and 0.8- fold synergistic factor to fenvalerate + PBO mixture from YG and SCD strain collected from China, respectively [49]. Enhanced monooxygenases were implicated in pyrethroid resistance in Australian *H. armigera*, based on evidence with PBO as a synergist [18]. Oxidases (MFOs) and esterases were found to be important mechanisms mediating pyrethroid resistance in *H. armigera* in India and Australia [22, 28]. Kranthi *et al*., reported that enhanced synergism by PBO was positively correlated with high levels of cytochrome P_{450} [29]. Mechanism of pyrethroid resistance in *H. armigera* varies across the different regions of India, but PBO (MFO mediated) found to be the major mechanism of imparting resistance in pest populations as reported by Armes [4]. When fenvalerate is mixed in combination with DEM at ratios of 1:2, 1:3 and 1:5 showed the synergistic factors of 1.10, 1.54 and 1.69, whereas, with TPP at ratios of 1:2, 1:3 and 1:5 showed the synergistic factors of 1.38, 1.92 and 2.20, in ASR-Sel strain, respectively TPP and DEM at different ratios showed slight synergism on fenvalerate in ASR-Sel strain wherein among the three different synergists PBO showed strongest synergism in the ratio 1:5 followed by 1:3 and 1:2 to ASR-Selected strain. McCaffery *et al*., found slight synergistic effect of DEF and TPP with pyrethroid insecticides in the population of *Heliothis virescens* [36]. Collins reported that DEF give minor synergism of predominantly oxidative pyrethroid resistance, whereas Perry *et al*., found TPP having partial synergistic activity [13, 40]. Gunning *et al*., attributed that resistance to pyrethroids is due to the overproduction of esterase, as implied by synergism study in *H. armigera* [21]. The results of present study suits with the findings of previous workers that synergism by PBO implied monooxygenases that might be involved in imparting resistance to pyrethroids in this pest.

The present study results indicated that all the ASR and ASR-Sel populations of *H. armigera* had significantly higher esterase activity than the SUS strain. The highest esterase activity was found in ASR-Sel strain (3.90 µmoles naphthol min^{-1} mg^{-1}) followed by ASR resistant strain (2.20 µmoles naphthol min^{-1} mg^{-1}) and SUS strain (0.98 µmoles naphthol min^{-1} mg^{-1}). The field populations ASR and ASR-Sel strain exhibited 2.24 and 4.0- fold higher esterase activity compared

to the SUS strain. The results are in conformity with Sangha *et al*., who reported 3.98- fold high level of esterase activity in resistant *H. armigera* under Punjab conditions as compared to susceptible strain [42]. Five-fold esterase activity in pyrethroid resistant strain of *H. armigera* was also reported by Huang and Han [23]. However, Manikandan and Ravisankar reported higher amount of esterase activity (1 to 9 fold) in pyrethroid resistant strain of *H. armigera* than susceptible population [33]. The present findings are different from the observations of Gunning *et al*., who reported 50 fold higher esterase activity in resistant populations of *H. armigera* as compared to susceptible strain [40]. He also observed that the resistance level in *H. armigera* was positively correlated with esterase titers and that increasing resistance was accompanied by increasing esterase activity. Cheema reported 3.7- fold higher esterase activity in fenvalerate selected strain of *Spodoptera litura* as compared to susceptible strain [10]. Wu *et al*., reported 1.90- fold higher esterase activity in YGF fenvalerate resistant strain of *H. armigera* in China Province compared to the susceptible strain [47]. Kranthi *et al*., also observed that enhanced esterase activity is an important mechanism in imparting resistance to pyrethroids [28]. Forrester *et al*., tested a range of pyrethroid structures against a pyrethroid-resistant strain of *H. armigera* with increased monooxygenses as the major metabolic mechanism [18]. Highest resistance factors were found to the ester-bonded phenoxybenzyl alcohol pyrethroids such as fenvalerate, cypermethrin, deltamethrin and cyhalothrin.

The activities of AChE in three populations were measured with acetylthiocholine iodide as a substrate, and the results are presented in (Table 6, Fig. 2). The AChE activities were found to be 3.88, 3.35 and 1.94 nmole min^{-1} mg^{-1} of protein in the ASR-Sel, ASR and susceptible strain, respectively. The specific activity of this enzyme in whole body homogenate of *H. armigera* was found higher in the ASR-Sel and ASR as compared to the susceptible (SUS) strain. The populations ASR-Sel strain and ASR exhibited 2.00 and 1.72- fold enzyme activity as compared to the SUS strain. Many authors reported variable results regarding the AChE activity in populations of different insects. Sangha reported an increase of 1.09 times in the specific activity of AChE in *H. armigera* from Abohar populations (8.61 nmole min^{-1} mg^{-1} of protein) as compared to that of Ludhiana region (7.89 nmole min^{-1} mg^{-1} of protein) [42].

The activity of glutathione S- transferase in whole body homogenates of *H. armigera,* as inferred from CDNB assay, was higher in the ASR and ASR-Sel as compared to the susceptible (SUS) strain. ASR-Sel recorded 1.70- fold enzyme activity (0.59) and ASR had 1.29- fold higher GST activity (0.45) than the susceptible strain (0.34 µmoles min^{-1} mg^{-1} of protein) (Table 6, fig. 2). Martin *et al*., reported 2.75-fold higher GST activity (0.55 µmoles min^{-1} mg^{-1} of protein) in the resistant strain as compared to the susceptible strain (0.20 µmoles min^{-1} mg^{-1} of protein) of *H. armigera* [35]. The data presented in Table 5 showed some synergism of fenvalerate with DEM (SR 1.69 in ASR-Sel strain). Thus from synergist experiments and enzyme assay studies, it can be inferred that there may be some involvement of GSTs in imparting the resistance in *H. armigera*. Similar results have been reported in *H. armigera* Yang *et al*., in *Nilparvata lugens*, Vontas *et al*., 2001 and in *Sitophilus zeamis*, Fragoso *et al*., [49, 46, 19].

Cytochrome P_{450} dependant monooxygenases/mixed function oxidase (MFOs) mediated detoxification is one of the most important mechanism of insecticide resistance. Due to the broad substrate spectra of MFOs, this mechanism may potentially affect several classes of insecticides [38]. The specific activity of the enzyme found to be highest in ASR-Sel (471.47 nmole min^{-1} mg^{-1}) and ASR (240.83 nmole min^{-1} mg^{-1}) compared to that of susceptible strain (SUS) which exhibited specific activity of (117.17 nmoles min^{-1} mg^{-1} protein), which was significantly lower than all other populations. MFO activity of ASR-Sel and ASR population of *H. armigera* was 4.02 and 2.05- fold, respectively as compared with susceptible population (Table 6, Fig. 2). It is likely that enhanced detoxification by MFOs is the major mechanism of pyrethroid resistance in *H. armigera* populations of Punjab. The study is in conformity with Ashraf *et al*., found 2.40 and 1.79- fold higher monooxygenase activity in resistant *H. armigera* population of Nagpur and Delhi, respectively compared to susceptible strain [7]. He also observed a strong positive correlation between monooxygenase activity and pyrethroid resistance and indicated that the elevated cytochrome P_{450} monooxygenase activity is associated with pyrethroid resistance in Indian strains of *H. armigera*. Similarly, Martin *et al*., reported that monooxygenase activities were identified to be the major metabolic mechanism responsible for resistance to pyrethroid [34]. Yang *et al*., who highlighted that P_{450} monooxygenases are a major metabolic mechanism responsible for pyrethroid resistance in *H. armigera* from India, China and Pakistan with combined evidence from synergism experiments, monooxygenases activity assays with multiple substrate and *in vitro* metabolism study [49].

A strong positive correlation (r = 0.99) between monooxygenases and pyrethroids resistance in *H. armigera* was observed. These results are in accordance with the studies conducted by Fahim *et al*.,[16]., who also observed the highly significant ($p<0.01$) correlation with synthetic pyrethroid in leaf dip method as well as in topical application method against *H. armigera* populations collected from Pakistan [16]. Similarly, Kranthi *et al*., who also observed a positive correlation between LC_{50} value of cypermethrin and general esterase activity in *P. xylostella* [28]. Silva *et al*., reported potential cross resistance among the synthetic pyrethroid and organophosphate insecticide tested against the cotton leaf worm [45]. Gunning *et al*., reported that resistance levels in *H. armigera* were positively correlated with esterase titers and that increasing resistance was accompanied by increasing esterase activity [21]. Owing to significantly high correlation, the activity of esterase can be used as a biochemical tool for monitoring of insecticide resistance in the pest. Similar studies conducted for pyrethroid resistance in *H. armigera* also showed higher MFO activity in the selected strain and strong positive correlation between MFO activity and pyrethroid resistance [9, 11]. Similarly Ashraf *et al*., observed a strong positive correlation between monooxygenase activity and pyrethroid resistance and indicated that the elevated cytochrome P_{450} monooxygenase activity is associated with pyrethroid resistance in Indian strains of *H. armigera* [7]. The present investigation indicating that enhanced detoxifying enzymes is playing major mechanism contributing to pyrethroids resistance in ASR and ASR-Sel strains. Biochemical tools in terms of activities of insect detoxification enzymes such as MFOs, GSTs and esterase can be used as means of insecticide monitoring. Furthermore, the information obtained on resistance mechanisms can serve as the basis for developing rational resistance management strategies.

5. Conclusion

The results revealed that the use of pyrethroids should be discouraged and in order to promote other insecticides and to postpone the development of resistance, a resistance management strategy of decreased selection pressure could be achieved by alternating these insecticides on the basis of proper pest scouting and pest status for decision of control application. Biochemical tools in terms of activities of insect detoxification enzymes such as MFOs, GSTs and esterase can be used as means of insecticide resistance monitoring. Furthermore, the information obtained on resistance mechanisms can serve as the basis for developing rational resistance management strategies.

6. Acknowledgments

The authors are thankful to the Professor and Head, Department of Entomology, Punjab Agricultural University, Ludhiana, India for providing the necessary research facilities.

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