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# Pyrethroid resistance and esterase activity in three strains of the cotton bollworm, *Helicoverpa armigera* (Hübner)

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ABSTRACT

Microplate assay technique for estimation of esterase activity in a single insect was used in combination with dose mortality bioassays to detect pyrethroid resistance in three strains of *Helicoverpa armigera* and to study the frequency of pyrethroid resistant individuals within the population of the same strain at two larval stages, third and fifth instar. The third and fifth instar larvae of the field strains *i.e.*, Nagpur strain and Delhi strain that displayed high degree of resistance towards deltamethrin, had higher esterase activity compared to a susceptible laboratory strain. The frequency distribution of individuals with elevated esterase activity above 1.00 absorbance unit was correlated with the resistance level of the strains. The frequency of resistant individuals in the third instar larvae of Nagpur strain and Delhi strain were 29% and 23%, respectively compared to 4% in the susceptible strain. The resistant individuals in the resistant strains have markedly increased in the fifth instar larvae with a frequency distribution of 63% and 90% in Delhi strain and Nagpur strain, respectively, while only 14% of individuals was found to have elevated esterase activity in the susceptible strain. The results demonstrated the role of esterase in pyrethroid resistance in *H. armigera*. Microplate assay proved to be a rapid and reliable biochemical technique for monitoring of pyrethroid resistance in *H. armigera*.



## 1. Introduction

The American bollworm, *Helicoverpa armigera* is one of the key pests having almost world-wide distribution. It is distributed in most of Asia, Australia, Africa and southern Mediterranean region, including 29 cotton producing countries such as India, China, Pakistan and Egypt [9]. It is recorded from 182 plant species including cotton, cereals, pulses, vegetables and fruits. It was estimated that out of $480 million spent on insecticides in agriculture in India annually, nearly 50% is used on cotton out of which 75% is used against *H. armigera* [19]. Chemical control, especially the use of pyrethroids is currently essential for management of *H. armigera* and is likely to remain an important component of control strategies in the future. However, the most serious threat to the continued effectiveness of pyrethroid in the field is the development of resistance. The resistance of *H. armigera* to pyrethroids was first reported in Australia [12] and later it was reported in many other countries. In India, pyrethroid resistance was first noticed between "1984 and 1985" in the southern region of India [8,23] and later it was reported in many other regions in the country. Kranthi et al. [18] conducted a survey comprising 54 field strains of *H. armigera* collected from 23 districts in seven states of India. All the 54 field strains studied were resistant to all four pyrethroids used (cypermethrin, deltamethrin, fenvalerate and λ-cyhalothrin). Exceptionally high resistance factors for deltamethrin (14,133- and 26,151-fold) were recorded for strains collected from Amaravati and Akola, respectively.

Different mechanisms of resistance to pyrethroids have been identified in *H. armigera*, including enhanced metabolism [3,22], nerve insensitivity [13] and reduced penetration [1]. Among the known metabolic resistance mechanisms *viz.*, esterase and monooxygenase, overexpression of esterase has been extensively reported in *H. armigera* [14,20,15,27,29]. In this study, we conducted an esterase based biochemical assay using microplate method integrated with dose mortality bioassays to detect pyrethroid resistance in *H. armigera* and to study the frequency of pyrethroid resistant individuals within the population of the same strain.

## 2. Materials and methods

### 2.1. Insect culture

The larvae of the American bollworm, *H. armigera* (Hübner), were collected from different fields of cotton and chickpea from Delhi and Nagpur. The field collected larvae were transferred

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to laboratory and reared individually for several generations on chickpea-based semi-artificial diet according to Singh [26] with minor modifications. The diet content of Chickpea powder (110 g), dried yeast (active, 20 g), casein (fat free, 10 g), methyl-*p*-hydroxy benzoate (2 g), sorbic acid (0.5 g), ascorbic acid (2.6 g), cholesterol (0.115 g), streptomycin sulphate (0.1 g), multivitamin mixture (1.0 g), vitamin E (α-Tocopherol, 0.6 g), bacto-agar (12 g), formaldehyde (1 ml) and double distilled water (720 ml).

A pyrethroid susceptible laboratory strain of *H. armigera* which was maintained for last 15 years without exposing to any insecticide was obtained from Central Institute for Cotton Research, Nagpur. The $LD_{50}$ values of deltamethrin and cypermethrin to this strain were 0.001 and 0.007 μg/third instar larva (30–40 mg), respectively [17]. Esterase was reported as a major mechanism of pyrethroid resistance in both Nagpur and Delhi strains. Nagpur strain and Delhi strain were found to have 2.24- and 1.73-fold higher esterase activity, respectively, than that of the susceptible strain and also additional esterase isozymes which were not observed in the susceptible strain [2]. The field strains and the susceptible laboratory strain were maintained in the insectary under constant temperature (25 ± 2 °C), 70 ± 5% RH and a photoperiod regime of 14 h photophase and 10 h scotophase.

### 2.2. Insecticide bioassay

Technical grade deltamethrin (98.5%) was obtained from Gharda Chemicals Limited, Thane, Maharashtra, India. The third instar larvae (30–40 mg) and fifth instar larvae (100–120 mg) were sorted out based on their weight and were assigned for topical application. Serial dilutions of the insecticide were prepared in acetone and a dose of 1 μl (in case of third instar larvae) or 2 μl (in case of fifth instar larvae) of the different solutions were applied on the dorsal thoracic region of the larvae using a calibrated and pre-programmed micro-applicator (Stoelting Autogenic Systems, USA). Thirty larvae for each dose were treated and control larvae were treated with acetone. The treated as well as the control larvae were held individually under the same condition of the stock insects with sufficient quantity of diet. Mortality was recorded up to three days and the $LD_{50}$ was calculated according to Finney [11]. Resistance factors were calculated as the ratio of the $LD_{50}$ of resistant strain to the $LD_{50}$ of the susceptible strain.

### 2.3. Microplate assay of esterase activity

To demonstrate the frequency of resistant individuals present in the populations, esterase activity in individuals of the pyrethroid-resistant and susceptible strains was estimated using the microplate assay method described by Brogdon and Dickinson [7] and used to study pyrethroid resistance in mosquitoes [25] with minor modification. One hundred third instar (30–40 mg) and 100 fifth instar (100–120 mg) larvae from each strain were drawn from the colony. In case of third instar larvae, individuals were homogenized in 1 ml of potassium phosphate buffer (40 mM, pH 6.8) in Eppendorf tubes. In case of fifth instar, the larvae were dissected and the midgut of each larva was individually homogenized in 0.5 ml of potassium phosphate buffer (40 mM, pH 6.8). Aliquots of 90 μl (20 μl homogenate solution, 70 μl phosphate buffer) were used for each assay, in triplicate. To each of the 90 μl of the homogenate, was added 90 μl of α-naphthyl acetate (56 mg/10 ml acetone/90 ml phosphate buffer, pH 6.8) using 96 wells ELISA plate and was incubated at 30 °C for 10 min. Ninety μl aliquots of Fast blue RR dye (100 mg/100 ml phosphate buffer, pH 6.8) were then added and absorbance was read using interference filter of 550 nm in ELISA reader (Thermolab Systems, Finland, Model No. 352).

In order to precisely account for the variation between individual larvae, the protein content of each larval homogenate was determined using the method of Lowery et al. [21] and the esterase specific activity was calculated at OD 550 nm/mg protein/min. Esterase activity, as a frequency distribution of different strains, was plotted against absorbance values at 550 nm/mg protein/min. Frequency distribution graphs were generated using Mintitab 11.12 software.

## 3. Results

### 3.1. Insecticide bioassay

The susceptibility of the 3rd instar and fifth instar larvae of field strains of *H. armigera* towards deltamethrin was studied. Results of the dose–response relationships of the 3rd and 5th instar larvae to deltamethrin are presented in Table 1. The discriminating dose ($LD_{99.9}$ of susceptible strain) is 4.42 and 4.93 μg/larvae for 3rd and 5th instar, respectively. Both the field collected strains displayed high resistance ratios against deltamethrin in both larval stages compared to the susceptible strain. There were significant differences in the $LD_{50}$ values of the different strains based on non-overlapping fiducial limits. The $LD_{50}$ values highly increased in the fifth instar larvae compared to that of the third instar larvae in both Nagpur strain and Delhi strain. The pattern of pyrethroid resistance in this study is characterized by high $LD_{50}$ values combined with low slopes for ld-p (Log-dose *vs.* probit mortality) lines that suggest a wide variation in the susceptibility of individuals of the same strain.

### 3.2. Microplate assay of esterase activity

The frequency distribution of pyrethroid resistance within the population of the three strains of the test insect was studied based on esterase activity of individual larvae. The frequency distribution (in percent) of susceptible and resistant individuals was plotted against absorbance (OD) values at 550 nm/mg protein/min and the frequency of resistant larvae with elevated esterase activity was scored (Fig. 1 and Table 2). Both the resistant strains, Nagpur strain and Delhi strain were found to have elevated esterase

**Table 1**
Relative toxicity of deltamethrin against third and fifth instar larvae of different strains of *Helicoverpa armigera*.

| Larval stage | Strains | *df* | $\chi^2$ | Regression equation (*Y*) | $LD_{50}$ μg/larva (μg/g of larvae) | Fiducial limits | Resistance ratio |
|---|---|---|---|---|---|---|---|
| 3rd instar | Nagpur | 5 | 0.49479 | $Y = 3.62997 + 1.99550\ X$ | 4.85915 (138.83) | 3.88098–6.08386 | 6108.83 |
| | Delhi | 5 | *11.78208 | $Y = 4.97578 + 0.64576\ X$ | 1.09020 (31.15) | 0.83281–1.42709 | 1370.58 |
| | Susceptible | 4 | 1.07604 | $Y = 7.55685 + 0.82495\ X$ | $7.95430 \times 10^{-4}$ (0.022) | $4.56590 \times 10^{-4}$–$1.39159 \times 10^{-3}$ | 1.00 |
| 5th instar | Nagpur | 6 | 6.70843 | $Y = 0.50676 + 3.44662\ X$ | 20.12174 (182.92) | 16.76085–24.15705 | 2850.10 |
| | Delhi | 4 | 6.36328 | $Y = 1.40404 + 3.22728\ X$ | 13.00880 (118.26) | 11.61567–14.59957 | 1842.61 |
| | Susceptible | 5 | 0.79438 | $Y = 7.33757 + 1.08666\ X$ | 0.00706 (0.064) | 0.00478–0.01042 | 1.00 |

*Y*, Probit kill; *X*, Log dose; third instar, 30–40 mg body weight; fifth instar, 100–120 mg body weight.
* Significant at $P = 0.05$.

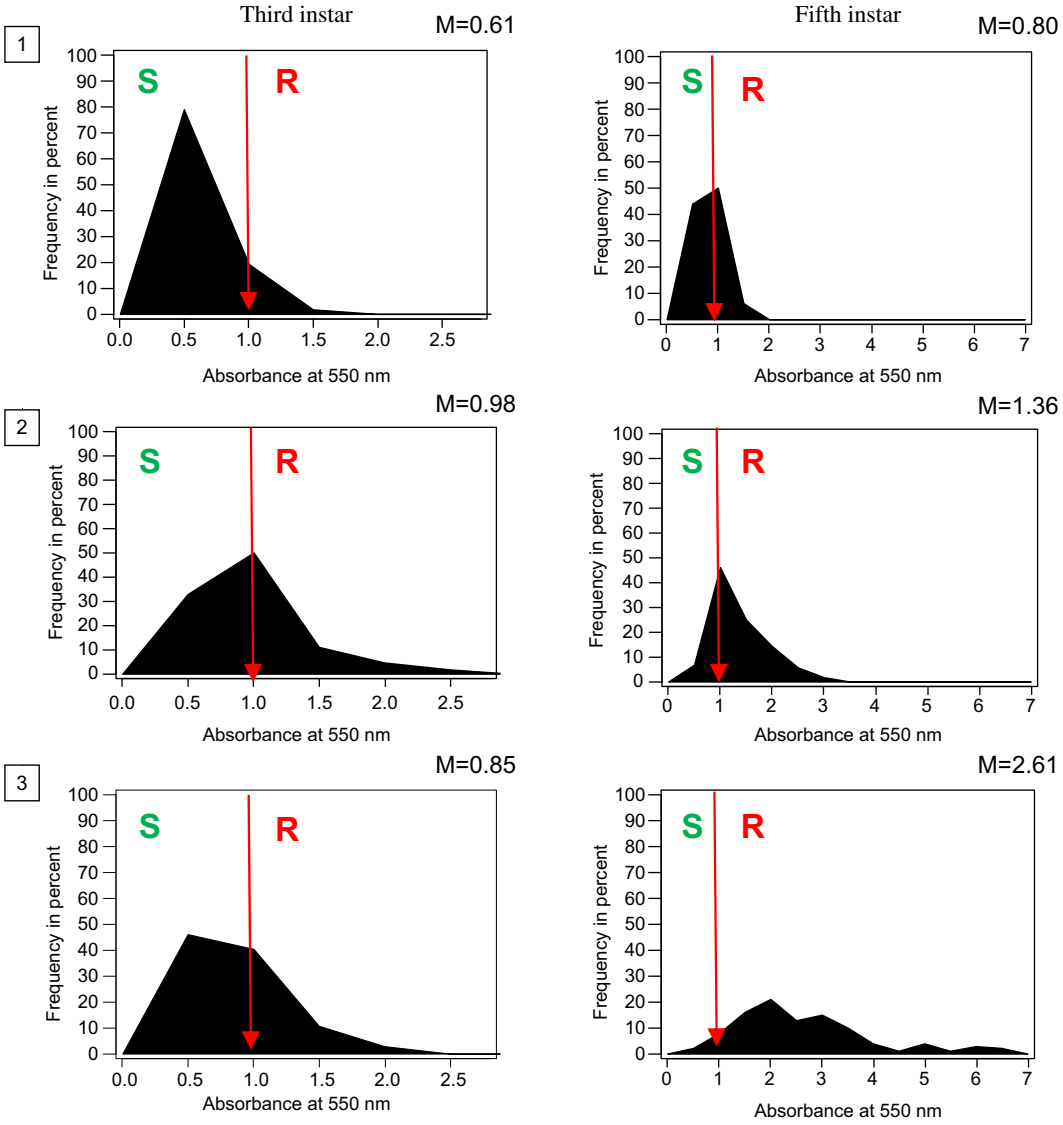


**Fig. 1.** Frequency distribution of esterase activity in the third and fifth instar larvae of susceptible, Delhi and Nagpur strains. (Data is based on esterase activity of whole body homogenate of third instar and midgut homogenate of fifth instar, $n = 100$). Absorbance at 550 nm = Absorbance at 550 nm/mg protein/min. 1, susceptible strain; 2, Delhi strain; 3, Nagpur strain; M, mean esterase activity of 100 individual. S, susceptible individuals; R, resistant individuals.

activity in comparison to the susceptible stain. Hence the mean of esterase activity in susceptible strain was 0.61 ± 0.22 and 0.80 ± 0.03 absorbance units (AU) for third and fifth instar, respectively. A threshold level of elevated esterase of 1.0 AU and above was considered as resistance threshold in all the three strains and both larval instars.

In the third instar larvae, esterase profile of the susceptible strain is characterized by the presence of a single peak of 0.5 AU with 80% frequency. The esterase activity within the population ranged from 0.29 to 1.44 AU with a mean of 0.61 ± 0.22 AU. Only 4% of individuals examined were found to have elevated esterase activity above 1.00 AU.

The esterase profile of the Delhi strain is characterized by the presence of a single peak of 1.0 AU with 50% frequency. The Delhi strain had a very wide range of esterase activity that ranged from 0.30 to 2.68 AU with a mean of 0.98 ± 0.05 AU. The frequency of individuals with elevated esterase activity was 29%.

In the Nagpur strain, 45% of individuals examined have esterase activity of 0.5 AU. The esterase activity varied from 0.30 to 1.91 AU with a mean of 0.85 ± 0.03 AU. The frequency of individuals with elevated esterase activity above 1.0 AU was 23%.

Comparing the three strains, Delhi strain had a higher esterase activity followed by the Nagpur strain and the susceptible strain with mean esterase activities of 0.98 ± 0.05, 0.85 ± 0.03 and 0.61 ± 0.22 AU for Delhi, Nagpur and susceptible strains, respectively. Although there was no marked difference between the three strains in terms of mean esterase activity, the frequency of individuals with elevated esterase (resistant individuals) in Delhi strain (29%) and Nagpur strain (23%) was 6- to 7-fold higher than that of the susceptible strain (4%). The frequency of resistant individuals in the Delhi strain (29%) was merely 6% higher than that of the Nagpur strain (23%).

In the case of the fifth instar larvae, both susceptible and Delhi strains had a single peak of 1.0 AU with a frequency of 48% and 44%

**Table 2**
Frequency distribution of esterase activity in the third and final instar larvae of susceptible, Delhi and Nagpur strains.

| Strain | | Mean ± SE (OD at 550 nm/mg protein/min) | Minimum OD | Maximum OD | % Resistant individuals[a] |
|---|---|---|---|---|---|
| Third instar | Nagpur | 0.85 ± 0.03 | 0.30 | 1.91 | 23.00 |
| | Delhi | 0.98 ± 0.05 | 0.30 | 2.68 | 29.00 |
| | Susceptible | 0.61 ± 0.22 | 0.29 | 1.44 | 4.00 |
| Fifth instar | Nagpur | 2.61 ± 0.13 | 0.58 | 6.69 | 90.00 |
| | Delhi | 1.36 ± 0.05 | 0.60 | 2.92 | 63.00 |
| | Susceptible | 0.80 ± 0.02 | 0.31 | 1.44 | 14.00 |

[a] Resistant individuals are the individuals that have absorbance (OD at 550 nm/mg protein/min) above 1.00.

in case of susceptible and Delhi strains, respectively. The esterase profile of Nagpur strain is characterized by the presence of four peaks at 2.0, 3.0, 5.0 and 6.0 AU with frequency distribution of 25%, 15%, 3% and 4%, respectively. In the susceptible strain, esterase activity ranged between 0.3145 and 1.5556 AU with mean of 0.80 ± 0.03 AU. The esterase activity of Delhi strain ranged from 0.60 to 2.92 AU with mean of 1.36 ± 0.05 AU. Nagpur strain had a very wide range of esterase activity *i.e.*, from 0.58 to 6.69 AU with a higher mean of 2.61 ± 0.13 AU.

The mean esterase activity in Nagpur and Delhi strains was 1.70- to 2.26-fold higher than that of the susceptible strain. Ninety percent of individuals in Nagpur strain were found to have elevated esterase activity compared to 63% in Delhi strain. While the frequency of resistant individuals in the susceptible strain, was only 14%.

There was 2- to 3-fold increase in esterase activity and frequency of individuals with elevated esterase activity in Nagpur and Delhi strains in fifth instar larvae compared to the third instar larvae. The frequency of resistant individuals had increased from 23% in third instar to 90% in the fifth instar in case of Nagpur strain and from 29% to 65% in case of Delhi strain.

## 4. Discussion

Esterase involvement in pyrethroid resistance is well documented. Gunning et al. [14] found that the pyrethroid resistant strains of *H. armigera* in Australia were having 50-fold increase in esterase activity compared to susceptible strain and also having additional esterases that were not detectable in the susceptible strain. Elevated esterase activity was reported to be a major mechanism of pyrethroid resistance in many other insect species *e.g.*, *H. zea, Spodoptera frugiperda, Agrotis ipsilon* [28] *Trichoplusia ni* [16], *Blattela germanica* [4]. Using microplate assay, [25] reported that elevated esterase activity is responsible for pyrethroid resistance in *Culex quinquefasciatus*.

In the current study, high pyrethroid resistance ratio was reported in the field collected strains of *H. armigera*. Pyrethroid resistance was combined with elevated esterase activity. The frequency distribution of esterase activity within the individuals of the same strain revealed that all the strains have a mixture of susceptible and resistant individuals based on esterase activity. The frequency distribution of individuals with elevated esterase activity was correlated with the resistance level of the strains. In the third instar larvae, the frequency of resistant individuals with elevated esterase activity above 1.0 AU in the resistant strains, Nagpur strain and Delhi strain were 29% and 23%, respectively compared to 4% in the susceptible strain. The fact that the third instar larvae of Delhi strain which have more resistant individuals than Nagpur strain, is less resistant to deltamethrin compared to Nagpur strain may be due to the involvement of another mechanism of resistance as elevated monooxygenase activity was also reported in both strain (unpublished data). The frequency of resistant individuals in the resistant strains has markedly increased in the fifth instar larvae with a frequency distribution of 63% and 90% in Delhi strain and Nagpur strain, respectively, while only 14% of individuals was found to have elevated esterase activity in the susceptible strain.

The wide range of esterase activity recorded within the individuals of the same strain reveals the wide variation in the susceptibility of individuals of the same strain which synchronize with low slopes for ld-p lines observed in the insecticide bioassay.

Microplate assay is considered as an excellent tool to identify resistance mechanism and to provide information on the frequency of resistant individuals present in the population [6]. Microplate assay was used to detect the presence of elevated esterase and confers resistance to insecticides in different species of mosquitoes [10,24,5,25].

The conventional methods of insecticide resistance involves insect rearing and topical application of insecticide is time consuming, labor intensive and results are not available immediately and are sometime unreliable. The results of the current study support the possibility of using microplate assay as a rapid and reliable biochemical technique for monitoring of pyrethroid resistance in *H. armigera*.

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