Effect of dihydrodillapiole on pyrethroid resistance associated esterase inhibition in an Indian population of *Spodoptera litura* (Fabricius)

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ABSTRACT

Resistance in *Spodoptera litura* (Fabricius) has been attributed to enhanced detoxification of insecticides by increased levels of esterases, oxidases and/or glutathione S-transferases. Enzyme inhibiting insecticide synergists can be employed to counter increased levels of such enzymes in *S. litura*. Dihydrodillapiole induced synergism of pyrethroid toxicity was examined in the laboratory-reared third instar larval population of *S. litura* collected in Delhi (susceptible), and Guntur (resistant) region of Andhra Pradesh, India. The Guntur population was found to be 7.04 and 10.19 times resistant to cypermethrin and lambdacyhalothrin, respectively. The activity of cypermethrin, lambdacyhalothrin and profenophos against susceptible and resistance populations of *S. litura*, was gradually increased when used along with a plant-derived insecticide synergist dihydrodillapiole. The α-naphthyl acetate hydrolysable esterase activity in Delhi population was less as compared to the Guntur population. Resistance associated esterases in Delhi population were inhibited by pre-treatment with dihydrodillapiole. The esterase level in insect was instantly reduced initially, sustained for about 3 h and equilibrated at 4 h post treatment. The esterase activity of Guntur population was increased to 1.28 μmoles/mg/min at 2 h post treatment and subsequently reduced to lower than 0.70 μmoles at 4–12 h post treatment. The variation in esterase activity is suggestive of its homeostatic regulation in test populations. Dihydrodillapiole thus caused significant reduction of resistance in *S. litura* to cypermethrin, lambda cyhalothrin and profenophos.

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1. Introduction

Spodoptera litura (Fabricius) (Lepidoptera: Noctuidae) is an important polyphagous insect pest infesting cotton, vegetable, oilseed and fiber crops. For emergency control of *S. litura* and other agricultural insect pests, synthetic pyrethroids and organophosphorus group of insecticides have been increasingly used. The insect over the years, has developed resistance to benzene hexachloride (BHC) [1], lindane and endosulfan [2], quinalphos and monocrotophos [3], and synthetic pyrethroids [4,5]. The outbreak of *S. litura* has been more common in the Indian sub-continent than any other part in the world [4–7]. Insecticide resistance is mainly attributed to decreased penetration, and enhanced detoxification by mixed function oxidases (MFO), esterases and glutathione-*S*-transferases. Among the detoxifying enzymes, enhanced esterase activity is one of the important pyrethroid resistance mechanisms in *Helicoverpa armigera* (Hubner) [4], *Spodoptera littoralis* [8], *S. litura* [6,9], and other insect pests [10]. One of the

ways to enhance the toxicity of insecticide is to use non-toxic synergistic compounds along with the insecticide [11].

Dihydrodillapiole (5-*n*-propyl-6,7-dimethoxy-1,3-benzodioxole), is a stable insecticide synergist derived from dillapiole, the major constituent of *Anethum sowa* seeds. Dillapiole, dihydrodillapiole, and semi-synthetic compounds based on them are known for their insecticide synergistic properties [12–13]. The comparable synergistic activity of dihydrodillapiole and piperonyl butoxide (PBO) [14–16] has brought attention towards the utility of plant based synergist dihydrodillapiole in insect pest management. Some other potential dillapiole based insecticide synergists against *Tribolium castaneum* (Herbst) include nonenoyl dihydrodillapiole [15], furapiole [15–17] and benzo-1,3-diole oxime *N-O*-butyl ether [17], with factor of synergism ranging from 4.5 to 6.3 compared to 2.2 for piperonyl butoxide (PBO). Dihydrodillapiole and related compounds also exhibit synergistic activity in *T. castaneum* with the carbamate group of insecticides [17].

The field population of *S. litura* from Guntur has been reported to be resistant to synthetic pyrethroids and the organophosphorus group of insecticides [4,18,19]. In this paper we report the effect of dihydrodillapiole on the synergistic and esterase activity of cypermethrin, fenveterate, lamda cyhalothrin, and profenofos against susceptible (Delhi) and resistant (Guntur) populations of *S. litura*.

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2. Materials and methods

2.1. Test insect

The egg masses of *S. litura* were collected from the cauliflower fields of Indian Agricultural Research Institute, New Delhi, and Peddavadalapudi in Guntur district of Andhra Pradesh, India. The rearing of the insect was done under laboratory conditions on tender castor (*Ricinus communis*) leaves under controlled conditions at 27 ± 1 °C and 60 ± 5% RH.

2.2. Test chemicals

The technical grade cypermethrin [α-cyano-3-phenoxybenzyl-*cis, trans*-3 (2,2-dichlovinyl)-2,2-dimethyl cyclopropane carboxylate] (93.0%), and fenvalarate [α-cyano-3-phenoxybenzyl 2-(4-chlorophenyl)-3-methyl butyrate] (92.5%) was procured from Rallis India limited, Mumbai. Lambda-cyhalothrin [(R+S) α-cyano-3-phenoxybenzyl (1S+1R) *cis* 3-chloro-3,3,3-tritrifluropropyl-1-enyl-2-2-dimethylcyclopropane carboxylate] (93.2%) and profenophos [*O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-propyl phosphorothiolate] (89.0%) were obtained from Syngenta India Limited, Mumbai. The technical grade dihydrodillapiole (100%) was obtained from Division of Agricultural Chemicals, IARI, New Delhi. α-Naphthyl Acetate (α-NAA) was procured from Sigma, USA. Acetone was used for the preparation of stock solutions.

Dihydrodillapiole
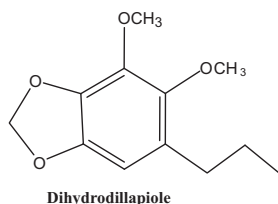
2.3. Treatments

Toxicity of cypermethrin, fenvalerate, lambda cyhalothrin and profenofos, alone and in combination with dihydrodillapiole was determined against third instar *S. litura* (25–30 mg) (Delhi and Guntur populations). The stock solutions (20%) were diluted to maintain the level of solvent (acetone) and emulsifier (Triton-X 100) at 5.0% and 0.5%, respectively in the final concentrations. A mixed formulation of each of the insecticide with dihydrodillapiole in four ratios (1:1, 1:2, 1:5, 1:10) was prepared by mixing the two stock solutions of equal concentrations. Test solutions of desired strength were made by serial dilutions of the stock solutions with water. Castor leaf discs of approximately 6 cm diameter. were dipped in the required concentrations of insecticides or insecticide + synergist combination for twenty seconds and then dried. The treated leaf discs were then transferred to clean jars (15 × 10 cm). In each jar 15 larvae were released and the jars kept at 27 ± 1 °C.

2.4. Bioassay

The larval mortality was recorded 24 h after the treatment. There were three replications for each concentration and untreated control. SAS [20] version 9.2 was used to calculate the probit and the regression analysis was performed simultaneously. Synergistic and antagonistic action of a particular combination of insecticide and synergist was calculated as per Sarup et al. [21].

2.4.1. Dihydrodillapiole treatment and extraction of esterase

Third instar larvae of *S. litura*, weighing 25–30 mg were sorted out from the rearing jar and kept separately for preconditioning at ambient temperature. In the *in vivo* esterase assay 1 µl of dihydrodillapiole (30 mM) in acetone was applied by a micropipette to the dorsal thorax of the test larvae. Samples of insects were obtained for esterase assay at two-hour intervals after treatment with dihydrodillapiole. The control group was not treated with synergist. Following the treatment, larvae were kept at a constant temperature of 27 ± 1 °C with adequate food for varying periods of time up to 24 h. For the extraction of esterase, the procedure developed by Kranthi [10] was followed. Thirty insects in each treatment were mass homogenized in 2 ml buffer (100 mM phosphate buffer, containing 1 mM each of EDTA, PTU, PMSF and 20% Glycerol, pH 7.0), and the homogenates were subjected to centrifugation at 10,000 rpm for 20 min. The volume of the supernatant obtained from centrifugation was made up to 2 ml using phosphate buffer (100 mM, pH 7.0). Hundred microliters of aliquot was taken from the supernatant in a 1.5 ml microcentrifuge tube and the volume made up to 1 ml. This solution was named as enzyme assay solution.

2.4.2. Esterase activity

Esterase activity was determined as per the procedure given by Kranthi [10]. Fifty microliters of enzyme assay solution was taken in a 10 ml test tube and the volume made up to 1 ml with 950 µl phosphate buffer (40 mM, pH 6.8). Five milliliters of substrate solution (1 ml of 30 mM α-NAA in 99 ml of phosphate buffer, 40 mM, pH 6.8) was then added to each test tube. One milliliter of 40 mM phosphate buffer with 5 ml of substrate solution without the enzyme assay solution was kept as control. The whole set was maintained in dark for 20 min at 30 °C with occasional shaking. After incubation, 1 ml of staining solution (2 parts of 1% fast blue BB solution in 5 parts of 5% SDS) was added to each tube including control and the tubes were kept in dark for 20 min at room temperature. 1-Naphthol produced as a product during the esterase action on the substrate (α-naphthyl acetate) was coupled with fast blue BB salt (Sigma, USA). A strong blue color produced was measured at its absorbance maxima of 590 nm on a double beam spectrophotometer (Perkin Elmer λ 3B). For the calibration of 1-naphthol, the procedure of Van Asperen [22] as detailed by Kranthi [10] was followed. Enzyme inhibition was expressed as the mean percentage of activity remaining (with respect to an un-inhibited control) for dihydrodillapiole. Three individual assays of esterase activity were made for each time interval. The standard error of mean was calculated for each replication and the error bars were drawn on the basis of upper and lower confidential interval. In view of the possible inhibition of esterase [23] by PMSF during homogenization, further study is required to confirm its role as an esterase inhibitor.

3. Results and discussion

3.1. Bioefficacy

The insect toxicity (LC_{50}) of cypermethrin, fenvelerate, lamda cyhalothrin and profenophos against *S. litura* 3rd instar larvae (Delhi and Guntur) is reported in Table 1. As evident from the data, the respective LC_{50} of cypermethrin, fenvelerate, lamda cyhalothrin and profenophos was 165, 311, 186, and 635 ppm in Delhi population; and 1162, 1432, 1896 and 149 ppm in Guntur population. As compared to Delhi population, Guntur population was more resistant to the insecticides.

Following application of insecticide–synergist combinations, significant increase in mortality has been observed in both Delhi and Guntur populations. The extent of synergism (synergistic ratio, SR) was calculated based on the LC_{50} of insecticide with out synergist/LC_{50} of insecticide with synergist. With increase in proportion of dihydrodillapiole in insecticide-synergist combination,

Table 1
Relative resistance of Delhi and Guntur population of S. litura of against different insecticides.

Insecticides	Heterogeneity[a] χ^2	Slope	LC$_{50}$ with out synergist (ppm)	Fiducial limits at 95% (ppm)	Relative resistance[b]
Delhi					
Cypermethrin	1.12	1.18	165	130–201	1:
Fenvalerate	3.88	0.94	311	188–525	1:
Lambdacyhalothrin	1.15	0.96	186	135–238	1:
Profenophos	1.78	1.22	635	463–946	1:
Guntur					
Cypermethrin	4.81	1.42	1162	799–1689	7.04
Fenvalerate	2.55	1.19	1503	810–2033	4.83
Lambdacyhalothrin	3.44	1.04	1896	902–2890	10.2
Profenophos	1.41	0.94	1490	254–6363	2.35

[a] In none of the cases, the data were found to be significantly heterogeneous at $P = 0.05$, Y = Probit kill, x = log concentration, LC$_{50}$ = Concentration (%) calculated to give 50% mortality.
[b] Relative resistance = LC50 Guntur population/LC50 Delhi population.

a gradual increase in insecticide synergism has been observed. Fenvalerate was however, an exception in Delhi population where no significant increase in toxicity was recorded. In Guntur population there was an increase in synergistic factor with increase in proportion of dihydrodillapiole with all the insecticides (Table 2).

3.2. Esterase activity

The esterase activity of Delhi and Guntur population of S. litura larvae with and without the insecticide synergist dihydrodillapiole is given in Fig. 1A and B. The esterase activity of the Delhi population varied between 0.768 and 1.104 μmoles of α-NAA/mg tissue/min in the 24 h of study. Notable variation was observed at two stages, the first between 0 h and 2 h (1.046 and 0.833 μmoles/mg/min) and the second between 16 and 19 h (0.768 and 0.953 μmoles/mg tissue/min) after the application of synergist. On the other hand, the esterase activity of Guntur population varied between 1.39 and 1.794 μmoles/mg/min in the 24 h. As compared to Delhi population, the Guntur population showed 7.04-, 4.83-, 10.20- and 2.35-fold resistance to cypermethrin, fenvalerate, lambda cyhalothrin and profenophos, respectively which was relatively higher than the susceptible Delhi population.

Esterase activity profile of both the Delhi and Guntur populations following topical application of dihydrodillapiole is presented in Fig 1A and B. In the susceptible population, esterase activity was reduced in the first 3 h. The reduction was initially instantaneous, sustained for 3 h post treatment, and equilibrated at 4 h post treatment. At this stage, the esterase activity with and without synergist treatment was comparable in Delhi population. The instantaneous reductions in esterase activity following treatment with dihydrodillapiole, particularly in the more susceptible strain, may be due to an *in vitro* artefact on homogenisation, caused by free dihydrodillapiole on the insect cuticle. It was interesting to note that this equilibration was followed by a rapid rise in esterase activity in the subsequent 2 h duration to 1.296 μmoles/mg/min. Though the esterase activity subsequently reduced in the next 14–16 h; it was not as low as in the first 3 h post treatment. In contrast the activity of esterase in the resistant population increased within 3 h and this increase was almost double. The highest increase being 1.28 μmoles at 2 h post treatment. Subsequently, the activity was reduced to values lower than 0.70 μmoles at 4–12 h post treatment. The initial increase was followed by rapid decrease from 4–13 h and later a substantial increase. The esterase activity was inherently higher (26%) in the resistant Guntur population compared to the susceptible Delhi population at the beginning of the instar (0 h). It increased to 40% and 56% in 12 and 24 h old larvae, respectively. As compared to Delhi population, dihydrodillapiole remarkably suppressed the esterase activity in the resistant Guntur population. Though there was an initial increase in the enzyme activity 3 h after treatment, in the resistant population it was 55% lower over the untreated resistant population (0.5 μmoles/mg/min) during the next 10 h.

Resistance to synthetic pyrethroids was noticed within four to five years of their introduction [8]. This phenomenon was widespread in several lepidopteran species, especially in the bollworms of cotton and S. litura which is major polyphagous foliage feeder. In India pyrethroid resistance was first identified as a major problem in Guntur district of Andhra Pradesh in 1987 on cotton bollworms [24–26]. Extensive studies on the field population of H. armigera [4,26] and S. litura [3] have brought to focus that resistance levels in Helicoverpa armigera are as high as 23- to 8022-folds and 0.2- to 197-fold in S. litura to the most extensively used pyrethroid (cypermethrin) in Andhra Pradesh. Several mechanisms of pyrethroid resistance have been reported in Spodoptera litura (Fab.) [3,6,10] and Heliothis virescens (Fab.) [27]. The preponderance of ester hydrolysis has been clearly demonstrated in S. littoralis (Boisduval)

Table 2
Effect of different synergist insecticide ratios on Delhi and Guntur population of S. litura *(synergist was applied with respect to insecticide application).

Insecticide	LC$_{50}$ at varying proportion of dihydrodillapiole in ppm								
	1:0	1:1	SR	1:2	SR	1:5	SR	1:10	SR
Delhi									
Cypermethrin	165	143	1.15	122	1.35	109	1.52	91	1.82
Fenvalarate	311	211	1.48	168	1.85	315	0.99	381	0.81
Lambdacyhalothrin	186	146	1.27	99.7	1.79	104	1.86	75	2.48
Profenophos	635	452	1.40	379	1.68	379	1.68	267	2.38
Guntur									
Cypermethrin	1162	1000	1.16	698	1.67	532	2.18	470	2.47
Fenvalarate	1432	1283	1.12	908	1.58	731	1.96	524	2.74
Lambdacyhalothrin	1896	1655	1.15	1249	1.52	926	2.05	660	2.88
Profenophos	1490	769	1.94	595	2.50	578	2.58	572	2.60

Synergistic ratio (SR) = LC$_{50}$ without synergist/LC$_{50}$ with synergist.
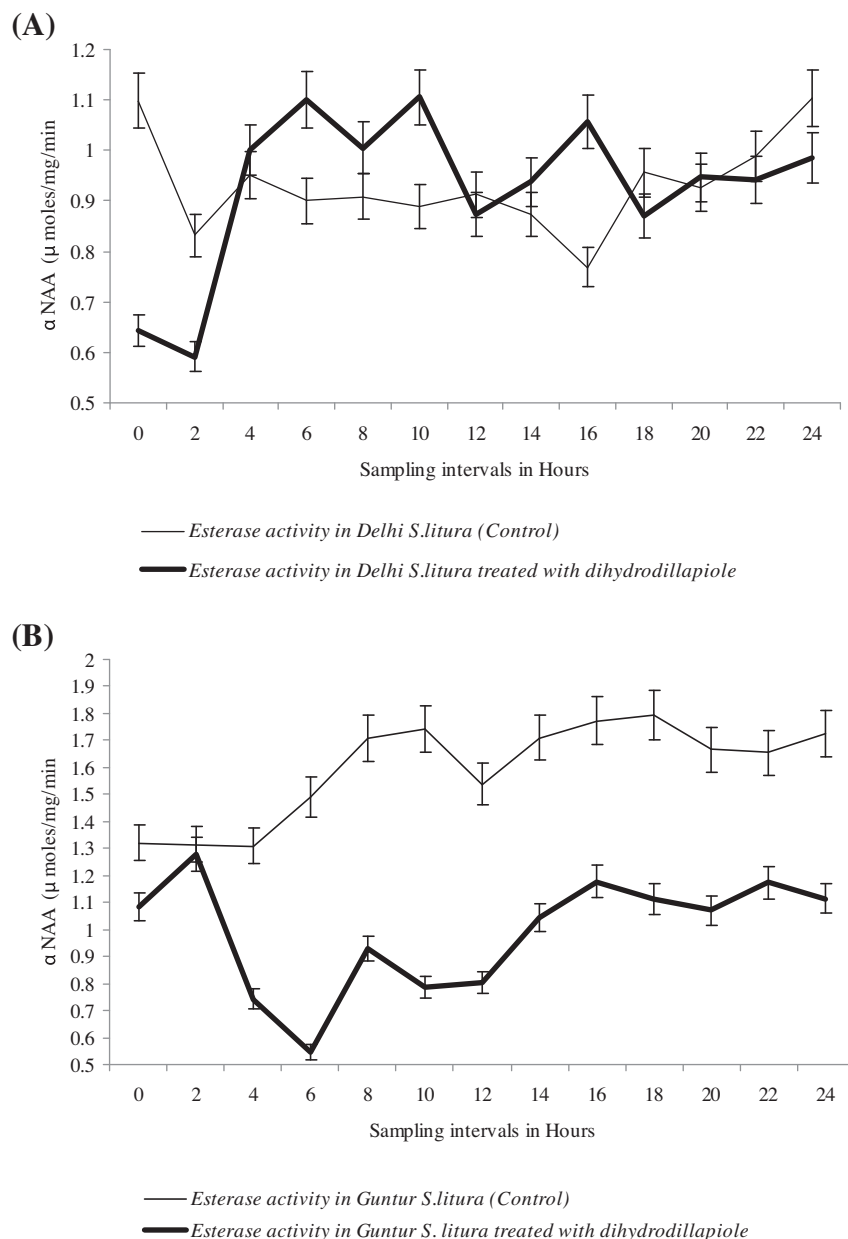
(A)

(B)

Fig. 1. Esterase activity of third instar *Spodoptera litura* larvae in (A) Delhi control and dhihydrodillapiole treated (B) Guntur control and dhihydrodillapiole treated.

[8] *S. litura* and *H. armigera* [10,28,29] and *Diabrotica virgifera virgifera* Howardi [30]. In the present study the increased esterase activity in the resistance Guntur population by 26% and its suppression by about 55% due to insecticide synergist dihydrodillapiole suggested the involvement of esterases in hydrolysis of cypermethrin. Though use of PMSF during homogenization and extraction of esterase may have inhibited some esterase activity [23] further work is required to look at this possibility. Young et al. [29] presented evidence to show the over production of esterase isozymes which metabolize and sequester pyrethroid insecticides in resistant *H. armigera* larvae. The study revealed that the classical synergist piperonyl butoxide (PBO) suppresses esterase activity 3–4 h post treatment and that the activity was regained gradually and reached to a normal level at 24 h. Similar results were reported in the whitefly *Bemicia tabaci* (Gennadius) (Aleyrodidae) [29]. The present study demonstrated the effect of dihydrodillapiole on esterase activity with an instant increase in activity within 2 h followed by esterase inhibition for the next 10 h and

later recovery to the normal level. Such a modulation of esterase activity was particularly noticed in the resistant population. However, there was marked deviation in the susceptible population wherein only initial suppression of the activity was noticed 4–5 h post treatment. Thus the esterase inhibition study has led to the conclusions that the process of initiation of synergism in the insect system starts with in 3–4 h and it extends up to 14 h. further the application of synergists prior to application of insecticide is more effective in inhibiting detoxifying enzymes and thus enhancing insecticide toxicity.

In previous studies, the resistant strains of *S. littoralis* have pyrethroid hydrolytic activity 3–6.5 times higher in resistant strain compared to susceptible strain [8]. Resistant strain of *Spodoptera frugiperda* showed at least 100 times higher LC$_{50}$ values than the susceptible strain. Besides delayed penetration, ester hydrolysis was a predominant mechanism responsible for resistance [31]. Ester hydrolysis has also been found to be a predominant mechanism of resistance in *Culex quniquifasciatus* (Say) but not in *Aedes aegypti*

(L.) [31]. The exceptionally high level of resistance to deltamethrin in field strain of *H. armigera* in different parts of India was attributed to the enhanced cytochrome-p450 and esterase activities [26].

4. Conclusions

Toxicity of insecticides in susceptible and resistance population of *S. litura* increased with increase in proportion of dihydrodillapiole in insecticide–synergist mixture. Esterase activity of the Delhi population varied between 0.768 and 1.104 μmoles of α-naphthyl acetate/mg/min in 24 h. The esterase activity of Guntur population on the other hand was high and varied between 1.3086 and 1.794 μmoles/mg/min. The inhibition of esterase activity by dihydrodillapiole in Delhi population was predominant in the initial 3 h post treatment, as against Guntur population. The study indicated that the application of synergists prior to application of insecticide may help in the inhibition of detoxifying enzymes and thus enhancing the toxicity of the insecticide. Dihydrodillapiole decreased the esterase activity from 4–13 h post treatment. Further analysis on the biochemical mechanism of resistance to pyrethroid insecticides in *S. litura* from different regions of India will be of immense physiological and agricultural importance.

Acknowledgments

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