Research Paper :

Influence of different hosts on induction of midgut carboxylesterase and cytochrome p-450 in *Helicoverpa armigera* (Hubner) and the effect on insecticide metabolism

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SUMMARY

Effect of different hosts *viz.* cotton, pigeonpea and chickpea of *H.armigera* on the induction of carboxylesterase and cytochrome p-450 enzymes were studied in the present investigation. Pigeonpea was found to be good inducer of CarE. Less induction of CarE activity was observed in larvae reared on cotton. Different isozymes were observed which showed significant difference in expression on PAGE. Pigeonpea strain showed maximum number of bands with dark intensity, followed by chickpea strain than by cotton strain. Microplate assay results showed higher expression of p-450 in pigeonpea strain with than chickpea strain. Less activity was shown by the cotton strain. Toxicity of different insecticides was tested against *H.armigera* reared on different hosts. The variability in toxicity was observed, and the strain reared on chickpea showed tolerance against indoxacarb, spinosad and emamectin benzoate, whereas, strain reared on pigeonpea showed higher LC_{50} for lambdacyhalothrin. Cotton fed larvae were found to be comparatively susceptible.

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Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae), is well known as cotton bollworm, gram caterpillar, pod borer or American bollworm. Highly polyphagous pest, *H.armigera* has great host-range from agronomical to horticultural crops with economic damage to a greater extent. It has been estimated that out of $ 480 million spent on insecticides in agriculture in India, nearly 50 per cent is used for cotton and out of that 75 per cent of this material is targeted against *H. armigera* (Kranthi *et al.*, 2001).

Indiscriminate use of chemicals against this pest led to emergence of serious problems of insecticide resistance to most of the common classes of insecticides. There are approximately 2000 active pesticide ingredients in use, most of which have been relied upon for many years. However, they act on about 25 physiological target sites. Highly efficacious insecticides with novel mode of action are becoming increasingly important in agriculture and currently essential for the resistance management in *H. armigera.* The latest generations of insecticides includes indoxacarb, emamectin benzoate, lambdacyhalothrin and spinosad and are found effective against lepidopteran pest like *H. armigera.*

Host plant can modify the susceptibility of herbivorous arthopods to pesticides (Yu,1986, Brattsten,1988). Plant species differ in the degree to which they stimulate the biochemical defense of insects. Therefore, research on insect host plant interactions may yield information of considerable value in the development of insect pest management programmes, where insecticides are an integral part of the programme (Berry *et al.*, 1980). Host plants may affect the expression of resistance to chemicals in lepidopteran pest like *Platynota idaeusalis,* tufted apple bud moth larval populations (Dominguez Gilly and McPheron, 2000). Host plant induces many biochemical components like enzymes, proteins in insect to detoxify chemicals,

xenobiotics. Induction of detoxifying enzyme by secondary plant substances in peppermint is observed (Berry *et al.*, 1980).

Detoxifying enzymes like CarE and MFO are the major factor in the host plant preference of phytophagous insects (Krieger *et al.*,1971) and there is now evidence of their induction by secondary plant substances which are ingested by insects in their diet (Brattsten and Wilkinson, 1977). These rise the possibility that plant species differ in the degree to which they stimulate such enzymes and that accordingly an insect ability to detoxify insecticides may depend on nature of its host plant.

The plant mediated herbivorous insect RNAi reported here lays the basis for new strategy, suppressing a critical insect gene (s) by feeding insects with plant tissue engineered to produce a specific dsRNA. There is a report on cytochrome expression in *Helicoverpa* that can be silenced through RNA interferences with transgenic plants. In future, this study will be helpful for use of advanced technique of gene silencing through RNA interference technology (Ying *et al.*,2007).

MATERIALS AND METHODS

Insects (*H. armigera*) were collected from field and reared in laboratory for homogenization on artificial diet (Armes *et al.*, 1992). There after larvae were reared for one generation on different hosts like cotton, pigeonpea and chickpea along with artificial diet. Commercially available formulations of indoxacarb, spinosad, emamectine benzoate, and lamdacyhalothrin were used in the present study for log dose probit (LDP) assay against 3rd or early 4th instar of *H.armigera.*

All chemicals used for enzyme and protein assay were purchased from Himedia Laboratories Ltd., Mumbai, India.

Laboratory rearing of *H.armigera:*

Field collected *H.armigera* was reared on artificial diet for 3-4 generations to develop homogenized population and escape field contamination. Last instar larvae were allowed to pupate in sterilized sand. Moth emerged from pupal chamber were subjected to mating chamber with 1male:1female ratio. Adult diet was given to moth. Egg laying was taken on muslin cloth. Neonates hatching from this cloth were taken on artificial diet

Rearing of *H.armigera* on hosts

Neonates from homogenous population were reared for one generation on plant parts of different host plants; *viz.*, squares and tender bolls of cotton, flowers and pods of pigeonpea, leaves and pods of chickpea. In next generation, neonates reared on same host till they reached to third instar. Bioassays were carried out with this population to determine the LC_{50} value of insecticides and effect of different hosts on susceptibility to insecticides in *H. armigera.* These all rearings were done under laboratory condition of 27±2^{0}C and 75±5% RH with 13:11 hrs light: dark period.

Enzyme preparation from midgut of *H.armigera:*

Randomly twenty larvae of late third or early fourth instar *H. armigera* weighing 300-350 mg approximately selected from F_1 of different hosts reared populations, *viz.*, cotton, pigeonpea, chickpea and diet. Larvae were starved for six to eight hours and chilled in refrigerator prior to dissection. The larvae were pinned dorsally at head and anal region in wax plate and dissected out with the help of sterilized dissecting needle in ice-cold sodium phosphate buffer (0.1 M, pH 7.0) containing KCl (1.15%). Midguts were isolated with removing adhered fat bodies. About a 10 midguts were placed in glass homogenization tubes containing 1 ml SPB (0.1mM pH 7.0 containing 0.1 mM EDTA, PTU and PMSF each). The homogenate thus obtained was centrifuged at 10,000 rpm for 15 minutes at 4°C in high-speed refrigerated centrifuge (Eppendrorf, Germany). Solid debris and cellular material was discarded. The resultant supernatant obtained was stored at –20°C and used for quantitative and qualitative analysis of protein and enzymes.

Quantification of detoxifying enzymes:

An indigenous microplate reader protocol was developed with modification in the protocols given by Dary *et al.* (1990) and Xu and Bull (1995) for CarE quantification. 14.42 mg a napthol dissolved in 5 ml acetone. Different concentrations were prepared from this stock with addition in SPB (0.04 M, 6.8 pH). Staining solution was prepared as 1 % Fast Brilliant Blue BB salt in SPB (0.04 M, 6.8 pH) and 5 % SDS in DDH$_2$O with 2 part FBB solution and 5 part SDS solution. All samples were estimated thrice, absorbance was taken on the microplate reader (Metertech model å960,USA) at 600 nm after 30 min. dark incubation at 30°C. Readings were plotted on graph to get standard curve of a napthol. For enzyme assay, substrate solution was a napthyl acetate (30 mM) dissolved in 1 ml acetone diluted with 10 ml SPB (0.04 M, 6.8 pH). 20 µl enzyme followed by 100µl freshly prepared substrate solution was added to wells of microplate with three replication. After half an hour incubation, 100 µl staining solution added to wells. After

half an hour incubation in dark absorbance was taken on microplate reader along with blank.

Cytochrome p-450 was analyzed with methods described by Brogdon *et al.* (1992). Cytochrome C from bovine heart was used to plot standard curve. 20 µl enzyme added in replicate in microplate followed by 200 µl of 0.0625 M potassium phosphate buffer pH 7.2, 200 µl of 3,3,5,5 tetramethyl benzodine (TMBZ) solution. TMBZ was prepared by dissolving 0.01 g TMBZ in 5 ml methanol with 15 ml of 0.25 M sodium acetate buffer pH 5.0. After these, 25 µl of 3 % hydrogen peroxide was added to each well. Control was taken with 20 µl buffer instead of enzyme. Plate was incubated for 2 hrs at room temperature and absorbance read at 450 nm on microplate reader. These values were compared with a standard curve of purified cytochrome C.

Qualitative study of carboxylesterase:

10 % SDS PAGE was performed under semidenaturing condition for carboxylesterase. Gel was pre-incubated in freshly prepared solution 100 ml SPB (0.04 M, 6.5 pH) with 0.02 % a napthyl acetate solution (20 mg a napthyl acetate in 2 ml acetone) for 20-30 min. Then gel kept in fresh staining solution with 1 % Fast Brilliant Blue BB salt and 0.02 % a napthyl acetate solution in 100 ml SPB (0.04 M, 6.5 pH) in dark at room temperature. To reduce nonspecific background staining of the gel, 2-4 ml of 4 % formaldehyde added to staining solution prior to staining (Kranthi, 2005).

Bioassay of *H.armigera* against insecticides:

3rd-4th instar larval population were reared on different hosts used for bioassay using leaf dip method under laboratory conditions. About one square cm. leaf disc of cotton leaf dipped in insecticide solution, allowed to air dry. 7-8 hrs starved larvae were released on that treated leaf disc in multicellular rearing tray. Five concentrations were taken with 10 larvae for each treatment with three replications. Control was also taken without insecticide. All the bioassays were carried out at 25±2^{O}C and 60-80% RH. Moribund larvae not responding to probing were considered as dead. Observations were recorded at 24, 48 and 72 hrs after treatment.

The median lethal concentration (LC_{50}) for the insecticides was worked out by using Indostat software, Hyderabad, India which is based on principles give by Finney (1977) and corrected mortality was calculated by using Abott's formula (1925).

Resistance ratio:

The resistance intensity of a population or a strain of insects to a particular insecticide is frequently quoted as the resistance ratio (RR), sometimes also called as resistance factor (RF) which was calculated by the formula :

$$RR = \frac{LC_{50} \text{ of host reared strain}}{LC_{50} \text{ of diet reared strain}}$$

RESULTS AND DISCUSSION

The results obtained from the present investigation have been discussed under following heads:

Induction of carboxylesterase and cytochrome p-450 by different hosts:

Higher expression of CarE in the strain reared on pigeonpea (350.99 nM mg protein^{-1} min^{-1}) with 3.55 fold increase over diet strain. Next to that, chickpea reared strain exhibits 289.72 nM mg protein^{-1} min^{-1} CarE activity having 2.94 fold higher activities over diet strain. Cotton imparted lower gut CarE enzyme activity which was only upto 193.07 nM mg protein^{-1} min^{-1} (Table 1).

Similar results were also obtained during CarE isozyme study, with notation E1 to E11 with relative front values. Diet strain showed only two isozymes having Rf values 0.33 and 0.45 with medium and light intensity, respectively. Pigeonpea strain showed maximum number of isozymes (8) followed by chickpea (6) and cotton (5). CarE isozyme with 0.25 Rf value showed medium intensity and all the isozymes with 0.35, 0.40, 0.43, 0.45, 0.48, 0.51 and 0. 58 Rf values with higher expression in pigeonpea strain (Fig. 1).

Table 1 : Induction of carboxylesterase and cytochrome p-450 in midgut of *H.armigera* reared on different hosts

Strain	Carboxylesterase		Cytochrome p-450	
	nM mg protein^{-1} min^{-1} (±SE)	Fold increase over diet	nM mg protein^{-1} min^{-1} (±SE)	Fold increase over diet
Cotton	193.07 (0.33)	1.96	193.07 (0.33)	1.96
Pigeonpea	350.99 (0.53)	3.55	350.99 (0.53)	3.55
Chickpea	289.72 (0.78)	2.94	289.72 (0.78)	2.94
Diet	98.40 (0.33)	-	98.40 (0.33)	-

Pigeonpea reared strain showed higher activity of p-450 up to 9.06 fold *i.e.* 119.52 µg ml^{-1} mg $protein^{-1}$ over diet strain 13.19. While chickpea and cotton reared larvae showed 103.83 and 85.89 µg ml^{-1} mg $protein^{-1}$ with 7.87 folds increase in activity

Toxicity studies:

Table 2 shows the toxicity data of different insecticides in *H.armigera* reared on different hosts. LDP assays showed that chickpea imparted higher resistance ratio of 8.81 in pigeonpea reared strain than artificial diet reared larvae. Chickpea reared strain showed resistance ratio of 4.51 fold and cotton strain found comparatively susceptible among different hosts studied in present investigation.

Cotton strain possesses lower resistance ratio (1.25 fold) against spinosad in comparison with diet strain. Chickpea was found to impart higher resistance (1.98 fold) in *Helicoverpa* followed by pigeonpea (Table 2).

LDP assay of emamectine benzoate in diet reared strain showed lowest (1.92 ppm) LC_{50}. Chickpea reared strain found resistant (2.34 fold) over diet reared strain. Pigeonpea reared strain exhibited resistance of 1.64 fold and cotton strain upto1.26 fold (Table 2).

Pigeonpea fed *H.armigera* larvae reported higher LC_{50} of lambdacyhalothrin about 30.13 ppm. Diet reared population of *H.armigera* showed LC_{50} 10.62 ppm. Chickpea strain showed LC_{50} of 24.55 ppm and cotton fed larvae found comparatively susceptible with 14.2 ppm LC_{50} (Table 2).

Interactions of host plant induced detoxifying enzymes in *H. armigera* and insecticide resistance:

Detoxifying enzymes study was found to play important role in resistance development in *H. armigera*. CarE reported to be induced in indoxacarb resistance in bollworm (Ghodki *et al.*, 2009), in *P. xylostella* esterase was found to be associated in indoxacarb resistance (Sayyed and Wright, 2006). Induced activity of various detoxifying enzymes by host plants reported to decrease toxicity of insecticide (Berry *et al.*, 1980).

Resistance in *H. armigera* to emamectin benzoate, avermectin group of insecticides, is found in chickpea reared strain than pigeonpea and cotton reared strain of *H.armigera*. The involvement of increased activity of CarE and MFO were reported by Lin et al (2009) in *Tetranychus cinanabarinus* against abamectin, avermectin group of insectcides resistance. Abamectin

Table 2 : Toxicity of insecticides in *H.armigera* reared on different hosts

Chemical	Strain	LC_{50} ppm (95 % FL)	LC_{95} ppm	Slope (±SE)	Chi-square	Resistant ratio
Indoxacarb	Cotton	2.45 (1.74-3.45)	17.47	1.92 (0.45)	0.203	2.37
	Pigeonpea	4.65 (3.35-6.44)	28.80	2.07 (0.50)	0.936	4.51
	Chickpea	9.08 (6.61-12.47)	56.60	2.08 (0.50)	0.729	8.81
	Diet	1.03 (0.86-1.24)	4.23	2.68 (0.54)	0.769	-
Spinosad	Cotton	21.24 (15.75-28.64)	132.48	2.06 (0.49)	0.229	1.25
	Pigeonpea	30.66 (23.18-40.57)	170.34	2.20 (0.53)	0.201	1.80
	Chickpea	33.81 (27.20-42.01)	165.07	2.38 (0.49)	1.61	1.9
	Diet	16.99 (12.60-22.91)	105.99	2.06 (0.49)	0.229	-
Emamectin	Cotton	2.42 (1.71-3.44)	18.58	1.86 (0.45)	0.118	1.26
benzoate	Pigeonpea	3.15 (2.40-4.15)	16.72	2.27 (0.54)	0.701	1.64
	Chickpea	4.50 (3.25-6.23)	29.71	2.00 (0.49)	0.608	2.34
	Diet	1.92 (1.41-2.61)	12.06	2 .04 (0.55)	1.22	-
Lambdacyhalothrin	Cotton	14.2 (11.01-18.42)	68.73	1.87 (0.45)	2.14	1.34
	Pigeonpea	30.13 (26.65-34.07)	66.0	4.83 (0.97)	0.18	2.83
	Chickpea	24.55 (20.16-29.90)	90.29	2.90 (0.69)	0.36	2.31
	Diet	10.62 (8.36-13.48)	69.09	2.03 (0.45)	3.96	-

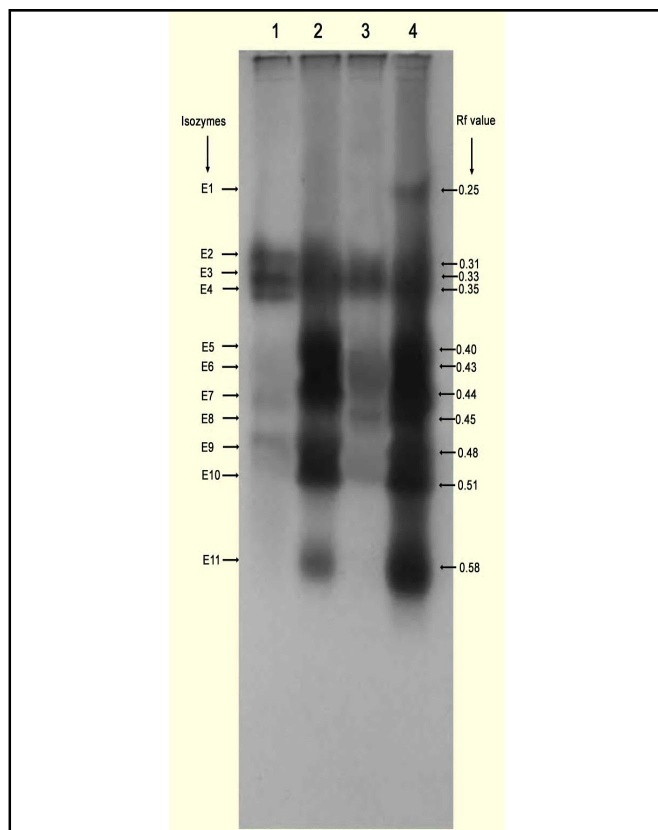

Fig. 1 : Esterase isozymes from of *H.armigera*
Lane 1- Cotton strain, Lane 2- chickpea strain, Lane 3- Diet strain and Lane 4- Pigeonpea strain

is closely related chemical with the emamectin. The present results also showed that chickpea imparted resistance to emamectin followed by pigeonpea, which might be due to induced activity for this induced activity of p-450 and CarE in *H.armigera*. Cytochrome p-450 involved in abamectin resistance in *B. tabaci* (Wang and Wu, 2007).

Spinosad metabolism involves the action of detoxifying enzymes like P-450 and CarE with variable amounts. In the present study, chickpea strain imparted higher resistance than pigeonpea strain followed by cotton reared strain. Chickpea was found to much induced activity of MFO and CarE next to pigeonpea. Spinosad resistance was found to be associated with increased titer values of CarE in *P. xyllostella* (Sudhakar,2005).

The present study showed that pigeonpea imparted resistance to pyrethroids through induction of CarE and MFO. Chavan (2001) and Tikar (1999) reported same results of pyrethroid resistance in pigeonpea fed larvae. Pyrethroid resistance in *S. litura* was found to be associated with enhanced activity of cytochrome p-450 monooxygenase (MFO) and esterase (Haung and Han, 2007), MFO and CarE was found to be involved in metabolism of synthetic pyrethroids in *H.armigera* (Ramsubramanian and Reghupathy, 2005). Availability of succession hosts to *H. armigera* leads to spread and maintenance of insecticide resistance (Duraimurugan and Reghupathy, 2005). Lambdacyhalothrin resistance was found to be governed by CarE, in red mites (Kumaral *et al.*, 2009). Report was also available that chickpea field collected strain of *H.armigera* showed low resistance to cypermethrin (Dhingra *et al.*, 1988).

Thus, the response of insects to insecticides may be greatly modified by the presence and concentration of host plant allelochemicals rather than being an independent phenomenon. This suggests that insects use the same system to defend themselves against dietary poison, insecticides and xenobiotics, allelochemicals.

The present investigation gives insight to mechanism of host-pest interaction with induction of detoxifying enzymes and insecticide susceptibility in *H. armigera*. Insecticide resistance development was found to be associated with host plant induction of detoxifying enzymes in *H.armigera*. The detoxifying enzyme induction and toxicity alteration in *H.armigera* by different hosts on which they feed, should not be neglected while suggesting pest management strategies. By understanding this mechanism of induction in insects, we can set novel targets for pest management which will be helpful to overcome insecticide resistance development

This will open new gate for research in areas which are related with insecticide resistance management. Alteration of toxicity in same insect species, feeding on different host plants specially in polyphagous pests is due to metabolic changes developed by insect while feeding on that particular host plants. Considering the immunity developed by insect against insecticides we need to modify the quantity of insecticides applied in field, as each host plays important role in imparting resistance against insecticides. It is due to induced levels of detoxifying enzymes in gut of insects.

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