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Effect of Nuclear Polyhedrosis Virus Infection on the Activity of Carboxylesterase and Glutathione S-Transferase in *Helicoverpa armigera* (Hbn)

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ABSTRACT. *Helicoverpa armigera (Hbn) is a polyphagous lepidopteran pest which has been reported to be resistant to many insecticides. One of the promising methods of management is microbial control with Nuclear Polyhedrosis Virus (NPV). Virus infection in H. armigera larvae is believed to increase susceptibility to insecticides. The present investigation undertaken to study the influence of virus infection on the activity levels of pesticide detoxifying enzymes namely carboxylase and glutathione S- transferase indicated significant decrease in the levels of both the enzymes in the larval body. The insecticides taken for the study were chloropyriphos and endosulfan. The insects treated with insecticides alone had increased levels of enzyme activity while viral infection alone resulted in lower levels of enzyme activity. When the insects were subjected to both NPV infection as well as insecticides the enzyme activity levels were extremely low. Possible reasons are discussed.*

INTRODUCTION

The gram pod borer *H. arimigera* is a polyphagous pest in India causing extensive losses in cotton, pulses, oilseeds and certain vegetable crops (Chari *et al.*, 1990). It has developed resistance to several pesticides (Armes *et al.*, 1994). But the nuclear polyhedrosis virus of (NPV) has been effectively used in the management of this pest on cotton, peanut, tomato, chickpea, sunflower and pigeonpea (Dhandapani, 1990; Dhandapani *et al.*, 1993; Narayanan and Gopalakrishnan, 1990; Rabindra and Jayaraj, 1988; Rabindra *et al.*, 1985; Venugopal Rao *et al* ., 1992).

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Studies (Rabindra and Jayaraj, 1990) have shown that the virus infection can increase the susceptibility of *H. armigera* larvae to endosulfan. One of the reasons for the susceptibility may be alterations in level of enzymes that are involved in metabolism of insecticides. Increased activity of carboxylesterases and glutathione S-transferase in insecticide-resistant population of *H. armigera* has been reported (Ibrahim and Ottea, 1995; Pasupathy *et al.*, 1994; Ramnath, 1990; Valencia *et al.*, 1993). Hence an investigation was made to assess the effect of HaNPV infection on the activity of these enzymes so that it may be utilized to mitigate development of insecticide resistance in *H. armigera*.

MATERIALS AND METHODS

Continuous culture of *H. armigera* was maintained in the laboratory to obtain uniform aged *H. armigera* for the experiments, which required 3rd instar larvae.

Optimum sublethal dose that would induce enzyme activity was to be standardized using serial dilution of LD_{99} values for endosulfan and chloropyriphos. There was increase in activity of enzymes at LD_{25} and LD_{50} both for endosulfan and chloropyriphos. However, when one more stress *i.e.*, virus infection was superimposed on treated larvae, the population treated with chloropyriphos at LD_{50} succumbed with in 72 h of superimposition of treatment. But the population at LD_{25} was able to withstand superimposed stress. So for chloropyriphos and endosulfan LD_{25} and LD_{50} respectively were taken.

The LD_{25} and LD_{50} values were freshly fixed each time during every test in order to avoid any inherent variations in the insect colony that may affect the response.

Assays on the activity of carboxylesterase and glutathione S-transferase were done as per the methods described below.

Carboxylesterase enzyme: extraction and assay

Carboxylesterase was assayed by following the method of Devonshire (1977). Four *H. armigera* larvae were homogenized individually in ice cold 20 mM phosphate buffer, pH 8.0 containing Triton X-100, using a pre-chilled glass pestle and mortar. For the extraction, 5 ml of the buffer

was used. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was separated and stored at 0-4°C.

Five ml of substrate solution (3×10^{-4} M α-naphthyl acetate containing 1 per cent acetone and 0.04 M of phosphate buffer pH 8; stock in acetone as 0.03 M) was mixed with 100 μl of homogenate. After 30 min incubation, 1 ml of coupling reagent was added. The change in absorbance was measured at 600 nm using spectrophotometer. A control, lacking enzyme extract was also maintained and its A600 was deducted from the sample. The amount of α-naphthol released by carboxylesterase was calculated using the standard graph. Total activity of enzyme was expressed as nmol of α-naphthol released per min per mg of protein. Quantification of protein was done by dye binding assay (Bradford, 1976).

Estimation of carboxylesterase activity in treated *H. armigera*

The insects were subjected to, treatment with chloropyriphos LD_{25}, inoculation with virus (HaNPV) LC_{50} and exposure to both virus LC_{50} + chloropyriphos LD_{25}.

Three tests were conducted. In the first test, 3rd instar larvae were treated with chloropyriphos. In the second test, the insects were treated with chloropyriphos and virus separately and the sampling was done after 72 h of treatment. In the third test, after 72 h of virus inoculation, chloropyriphos was applied. A set of untreated larvae was maintained as control in each experiment.

Insecticide treatment

Third instar larvae of *H. armigera* were used for the assay. One μl of chloropyriphos (technical grade) diluted with acetone to give LD_{25} dose (1.0 μg/larva - LD_{99}) was dispensed on the thoracic region of larvae and the larvae surviving after treatment were homogenized to extract the enzyme at specific time intervals of 3, 6, 12, 24, 48 and 72 h after treatment. For each time interval, 4 replications each with single larva were maintained.

Inoculation with virus

Third instar larvae of *H. armigera* were treated with LC_{50} dose of HaNPV by diet surface-contamination method. After 72 h of treatment, the larvae were separated from the pool for homogenization at specific intervals of 12 h to 132 h. Four larvae were used as replications per analysis. A set of control was maintained, without treatment.

Virus + chloropyriphos treatment

Third instar larvae of *H. armigera* were used for the assay. Larvae were allowed to feed on diet surface-contaminated with NPV at 1.8 POB/cm^2 (LC_{50} dose). After 72 h of treatment, the insects were given chloropyriphos LD_{25} dose. The larvae were used for homogenization at 12 h time interval till 72 h after treatment with insecticide. Four larvae were used as four replications per analysis. A set of control was maintained.

Glutathione S-transferase: extraction and assay

Activity (GST) Glutathione S-transferase was assayed by following the method of Kao *et al.* (1989). Single larva of *H. armigera* was homogenized in 5 ml of ice cold 0.2 M Tris-HCl buffer, pH 8 using pre-chilled pestle and mortar. The homogenate was centrifuged at 10,000 g for 10 min in a refrigerated centrifuge. The supernatant was collected and protein concentration determined following Bradfords method.

Two ml of GSH (glutathione, reduced:15 mg in 10 ml of 0.2 M Tris-HCl, buffer pH 8.0) solution was added to 0.2 ml of enzyme extract. Then 20 μl of 0.2 M CDNB (1013 mg 1-chloro 2, 4-dinitrobenzene dissolved in 2 ml distilled ethanol) in ethanol was added to sample, after the cuvette had been equilibrated to 34°C. Twenty μl of ethanol was added to the blank. The samples were incubated for 5 min and the change in absorbance measured at 340 nm using spectrophotometer. An extinction co-efficient of conjugated product 10 mM^{-1} cm^{-1} was used to calculate the specific activity.

Estimation of GST activity in treated *H. armigera*

The insects were subjected to, endosulfan LD_{50}, inoculation with virus (HaNPV) LC_{50} and exposure to both virus LC_{50} + endosulfan LD_{50}.

Three tests were conducted. In test one, 3rd instar larvae were treated with insecticide only. In test two, the insects were treated with insecticide alone and virus alone and sampling was done after 72 h. In test three, after 72 h of virus inoculation, endosulfan was applied. Suitable controls with untreated larvae was maintained. GST activity was measured as described above.

RESULTS

Activity of carboxylesterase

The activity of carboxylesterase in 3rd instar larvae increased in both healthy and insecticide-treated larvae, as they grew older. This increase in enzyme activity was significant after 6 h of treatment. The enzyme activity in chloropyriphos-treated larvae was higher than in control at all time intervals tested and the differences were significant at 6, 12 and 24 h (Table 1).

In the second test also, the carboxylesterase activity increased significantly with time and the differences between the insecticide-treated and control were significant up to 108 h post treatment. Although the carboxylesterase enzyme levels increased in the virus-treated insects as the larvae grew older, the levels were significantly lower than those treated with chloropyriphos and controls (Table 2).

However, when insects were treated with both insecticide and virus there was a decrease in enzyme activity which was significant beyond 36 h post treatment. The enzyme levels were significantly lower than those in larvae dosed with virus alone, starting with 36 h post treatment (Table 3).

Activity of glutathione S-transferase (GST)

The activity of GST in 3rd instar larvae of *Heliothis* increased significantly with age in both control and endosulfan-treated larvae (Table 4).

The activity was always higher in endosulfan treated insects than control insects and the differences were significant at all time intervals of treatment. Assays made 72 h onwards of treatment also showed a similar trend. However in the case of virus treatment, even though there was a marginal increase in the enzyme activity there were no significant differences

Table 1. Activity of carboxylesterase in chloropyriphos-treated and untreated larvae of III instar *H. armigera.**

Time (h) after treatment**	Enzyme activity n moles min^{-1} mg^{-1} of protein	
	Control	Chloropyriphos
3	423.14 e	413.26 e
6	447.30 d	470.12 e
12	562.16 c	678.04 d
24	692.43 b	892.36 c
48	720.01 b	987.72 b
72	861.43 a	1247.84 a

* Means of four determinations (each with duplicate incubations)
** Means followed by similar letters in a column are not significantly different (P=0.05)

Table 2. Activity of carboxylesterase in chloropyriphos and NPV treated larvae of *H. armigera.**

Time (h) after treatment $	Enzyme activity n moles min^{-1} mg^{-1} of protein**		
	control	chloropyriphos	NPV
72	991.10 d (B)	1130.16 e (A)	882.93 d (C)
84	1081.39 d (B)	1487.52 d (A)	984.31 c (C)
96	1587.60 c (B)	1801.54 c (A)	1184.12 b (C)
108	1763.21 b (B)	1956.16 b (A)	1264.23 ab (C)
120	2093.24 a (A)	2139.52 b (A)	1287.30 a (B)
132	2174.12 a (A)	2243.11 a (A)	1341.86 a (B)

* Means of four determinations (each with duplicate incubations)
** Means followed by similar capital letters in a row and small letters in a column are not significantly different (P=0.05)
$ All treatments were given simultaneously

Table 3. **Activity of carboxylesterase in larvae of *H. armigera* exposed to both chloropyriphos and NPV*.**

Time (h) after treatment	Enzyme activity n moles min^{-1} mg^{-1} of protein**			
	control	chloropyriphos	NPV	chloropyriphos + NPV ***
12	1063.32 e (B)	1289.06 f (A)	901.04 d (C)	876.72 a (C)
24	1254.17 d (B)	1408.32 e (A)	991.84 d (C)	840.32 ab (C)
36	1627.20 c (B)	1830.11 d (A)	1169.63 c (C)	872.46 a (D)
48	1788.32 b (B)	2044.26 c (A)	1231.32 bc (C)	753.51 bc (D)
60	1987.11 a (B)	2417.84 b (A)	1274.01 ab (C)	718.33 bc (D)
72	2102.36 a (B)	2601.91 a (A)	1304.81 a (C)	698.20 c (D)

* Means of four determinations (each with duplicate incubations)
** Means followed by similar capital letters in a row and small letters in a column are not significantly different (P=0.05)
*** Virus inoculation was done 72 h before the application of insecticide

Table 4. **Activity of glutathione S-transferase in endosulfan treated and control larvae of III instar *H. armigera**.**

Time (h) after treatment	Enzyme activity n moles min^{-1} mg^{-1} of protein**	
	control	endosulfan
3	34.62 c	46.24 d
6	37.74 c	49.30 cd
12	39.26 bc	52.11 c
24	41.18 bc	54.32 bc
48	43.78 b	59.62 b
72	55.31 a	64.03 a

* Means of four determinations (each with duplicate incubations)
** In a column, means followed by similar letters are not significantly different (P=0.05)

between 72 and 84 h as well as 84 and 132 h. The enzyme activities were always lower than those of control and endosulfan treated larvae (Table 5).

Table 5. Activity of glutathione S-transferase in endosulfan and NPV treated larvae of *H. armigera.**

Time (h) after treatment $	Enzyme activity** n moles min^{-1} mg^{-1} of protein		
	control	endosulfan	NPV
72	53.67 d	61.34 c	46.31 b
84	56.04 cd	64.39 bc	48.58 ab
96	59.32 bc	66.30 b	49.72 ab
108	62.78 ab	68.11 ab	51.33 a
120	64.33 a	69.31 a	50.47 ab
132	67.14 a	72.34 a	52.19 a

* Means of four determinations (each with duplicate incubations)
** Means followed by similar letters in a column are not significantly different (P=0.05)
$ All treatments were given simultaneously

When the insects were exposed to both the insecticide and virus, the enzyme levels were reduced further, much less than the levels seen in virus-treated larvae and significantly lower than that in control and endosulfan-treated insects at all time intervals tested (Table 6).

DISCUSSION

Carboxylesterases are ubiquitous, non-specific enzymes and have been implicated in the metabolism of diverse group of compounds including metabolism of lipids (Stevenson, 1969), xenobiotics (Mehrotra and Singh, 1976), and insecticides such as organophosphates, carbamates and pyrethroids (Plapp, 1971; Motoyama *et al.*, 1980; Abdel-Aal *et al.*, 1990). Carboxylesterases occur in a number of insect species and have been implicated as detoxifying enzymes responsible for organophosphorus

Table 6. **Activity of glutathione S-transferase in larvae of *H. armigera* exposed to both endosulfan and NPV*.**

Time (h) after treatment	Enzyme activity n moles min^{-1} mg^{-1} of protein**			
	control	endosulfan	NPV***	endosulfan + NPV$
12	55.92 d	62.78 e	49.29	40.95 a
24	59.36 cd	67.04 d	52.16	32.27 bc
36	63.96 c	72.36 c	50.33	34.12 b
48	67.14 bc	78.22 b	49.16	30.39 b
60	69.76 ab	84.30 a	48.37	28.34 c
72	74.33 a	87.92 a	48.04	29.18 c

* Means of four determinations (each with duplicate incubations)

** Means followed by similar letters in a column are not significantly different (P=0.05). Treatment means in a row are significantly different at all time intervals tested.

*** Differences among means are not significant.

$ Larvae were treated with virus 72 h before the application of the insecticide

insecticides (Sun and Chen, 1993; Sun *et al.*, 1987; Tranter *et al.*, 1984; Zhu and Brindley, 1992). These have been detected in *H. armigera* (Kuleiva *et al.*, 1994; Pasupathy *et al.*, 1994; Ramnath, 1990; Valencia *et al.*, 1993).

GSTs have been induced by numerous xenobiotics, including insecticides, drugs, host plants, and allelochemicals in over 21 species of insects. Among these inducers, insecticides (organochlorines), host plants (umbellifers and crucifers), and allelochemicals (furanocoumarins, indoles, and flavonoids) are the most potent inducers of the enzymes. Species differences in enzyme inducibility were observed in Lepidoptera. GST induction in insects was associated with increased GST mRNA levels indicating *de novo* synthesis of the enzyme. GSTs have been implicated in resistance to insecticides and allelochemicals in insects. The high GST activity found in insecticide-resistant insects was associated with increased level of specific mRNA (Kirby *et al.*, 1994; Yu, 1996).

In the present studies, the activity of carboxylesterase increased steadily with age. It is known that the enzyme activity increases rapidly with age (Behera, 1992). Changes in esterase titre during the larval developmental stages have been reported by Kapin and Ahmad (1980) in *Lymantria dispar* (Linnaeus). In *Lygus hesperus* (Knight), increase in esterase activity from II to V instar has been reported by Zhu and Brindley (1990).

The increase in esterase activity was also seen in larvae treated with sub-lethal dose of chloropyriphos (Tables 1-3). However, the levels were always higher than in untreated insects at all intervals. This elevated level of enzyme would probably enable the insects to detoxify the insecticide.

Population of *Helicoverpa virescens* (Fabricius) resistant to endosulfan, parathion methyl, deltamethrin and fenvalerate were found to contain higher levels of carboxylesterase activity than susceptible population (Valencia *et al.*, 1993). Insecticide resistant *H. armigera* population also showed a higher carboxylesterase activity than a susceptible laboratory population (Ramnath, 1990). Topical application of synthetic pyrethroids induced carboxylesterase activity in *H. armigera* populations showing high level of resistance (Pasupathy *et al.*, 1994).

In the present studies, the GST activity also increased with age of larvae though not as fast as in carboxylesterases. In endosulfan-treated insects there was a similar increase and the levels were higher than that in control insects at all periods suggesting that this population of *H. armigera* also responds to insecticides by increased level of enzymes.

GST activity has been found in larvae of *H. virescens* which was associated with insecticide resistance (Ibrahim and Ottea, 1995). GST activity has been studied in insects exposed to organophosphates (Chiang and Sun, 1993) and endosulfan (Egaas *et al.*, 1992). Increased GST activity was associated with BHC and cyfluthrin resistance in *Tribolium castaneum* (Herbst) (Cohen, 1986; Reidy *et al.*, 1990). When mites were exposed to fenpropathion, propoxur, chlordimeform, carbaryl and lindane, GST was induced (Capua *et al.*, 1991). GST was found to be the only mechanism which has been selected in *Phytoseiulus persimilis* Athias-Henriot, for metabolizing methidathion by resistant strains (Fournier *et al.*, 1987).

In the NPV-treated larvae also, there was an increase in the carboxylesterase activity with age but the levels were always significantly lower than those in healthy larvae (Tables 2 and 3). The effect of virus infection was more pronounced in the levels of GST. Even though there was

a marginal increase in the enzymatic activity with time, there was no significant differences between 72 and 84 h as well as 84 and 132 h. The GST levels in NPV-infected larvae were always lower than that in control as well as endosulfan-treated larvae (Table 5). These results indicate that the virus infection interferes with enzyme turnover. The virus multiplies primarily in the nuclei of the fat body as well as blood cells, apart from other tissues like the hypodermis and tracheal epithelium. Fat body is the chief site of protein synthesis (Shigematsu and Noguchi, 1969a) and any pathological condition of this organ should lead to a fall in the level of total proteins and hence the enzymes. Reduction in protein levels due to NPV infection has been reported earlier in several insects (Carstens *et al.*, 1979; Kelly and Lescott, 1981). Haemolymph protein fluctuates with intensity of infection and is known to decrease as infection progresses (Watanabe *et al.*, 1968) and the fat body, a major target of baculoviruses is a source of haemolymph proteins (Shigematsu and Noguchi, 1969b). Enzyme levels are known to fluctuate during virus infection (Shylaja and Ramaiah, 1984) and subsequently may be suppressed due to the destruction of fat body.

Sub-lethal infection by NPV was reported to increase the susceptibility of *H. armigera* larvae to fenvalerate, cypermethrin, endosulfan and monocrotophos (Rabindra and Jayaraj, 1990). This was probably due to suppression of detoxifying enzyme titre as a consequence of virus infection of the fat body.

The present studies indicated that, in the presence of virus infection in *H. armigera* larvae, there was no increase in the levels of carboxylesterase and GST enzymes due to insecticide treatment (Tables 2, 3, 5 and 6). On the contrary, when virus infected larvae were exposed to the insecticides, there was a slow but steady decline in the levels of both carboxylesterase and GST. These findings and reports indicate the scope of using viral pesticides for insecticide resistance management. The prospects of using HaNPV as a microbial insecticide for the management of *H. armigera* has already been discussed. Resistance to various insecticides has been reported in *H. armigera* (Ahmad *et al.*, 1995; Armes *et al.*, 1994). Cases of virus-induced susceptibility to insecticides have been reported earlier (Rud and Belloncik, 1984). Morris *et al.* (1974) showed that *Choristoneura fumiferana* Clemens NPV combined with fenitrothion caused the highest mortality. Synergistic action was reported when Elcar® was tested in combination with pesticides against *H. virescens* (Mohamed *et al.*, 1983). Ferron *et al.* (1983) found that low doses of specific NPV isolated from larvae of *Mamestra brassicae* (Linnaeus) and *Spodoptera littoralis* (Boisduval) together with a sublethal dose of deltamethrin caused synergistic effect when they were administered

simultaneously. The SmNPV in combination with quinalphos, fenthion and permethrin showed a synergistic action when tested against IV instar larvae of *Spodoptera mauritia* (Boisduval) (Mathai *et al.*, 1986). When a *M. brassicae* baculovirus was used in conjunction with deltamethrin against *H. armigera*, synergistic action was noted by Biache and Chaufaux (1986). In insecticide-resistant larvae of *H. virescens* and the sphingid *Manduca sexta* (Linnaeus) cytoplasmic polyhedrosis virus infection led to reduced cytochrome P-450 content and activities than those in control larvae and the resistance of *H. virescens* to insecticides disappeared (Brattsten, 1987).

The influence of sub-acute infection of polyhedrosis virus on the insecticide susceptibility has been reported in the cabbage looper *Trichoplusia ni* (Hubner) by Girardeau and Mitchell (1968) who postulated that devitalization of the host by a disease may so alter its physiology that stresses or toxins, relatively minor to a healthy insect, may have severe effects and even cause death in a diseased insect.

Breakdown of insecticide resistance due to infections by other microbial pathogens has also been reported. Justin *et al.* (1989) have reported that *Bacillus thuringiensis* (Bactospeine®) also increased the susceptibility of *H. armigera* and *Spodoptera litura* (Fabricius) larvae to insecticides. Increased insecticidal susceptibility due to protozoan infection has been reported in coleopteran insects like the American boll weevil, *Anthonomus grandis* Boh. (Bell and McLaughlin, 1970) and the flour beetles, *Tribolium confusum* Duval., *Tribolium destructor* Uytt. (Listov and Nesterov, 1976) and *T. castaneum* (Herbst) (Rabindra *et al.*, 1988).

CONCLUSIONS

The present studies have shown that the levels of carboxylesterase and Glutathione-S-transferase were significantly lower in NPV-infected larvae than those of their healthy counterparts. Obviously this is a result of infection of various tissues like fat body and blood cells which are important sites of protein metabolism. This finding explains why NPV infection increases susceptibility to insecticides in insects. By properly integrating use of NPV in IPM, the problem of insecticide resistance in *H. armigera* can be managed.

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