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Mitigation of insecticide detoxifying enzymes in *Plutella xylostella* using *Bacillus thuringiensis*

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ABSTRACT

The activity of insecticide detoxifying enzyme, carboxylesterhydrolase (CEH) and mixed function oxidase (MFO) were found to be increased in *P.xylostella* with or without à *priori* exposure to *B. thuringiensis* subsp. *kurstaki* (Delfin). A significant reduction was observed in the CEH titre and MFO titre in the population of *P. xylostella* pre-exposed to *B.thuringiensis* subsp. *kurstaki* (Delfin) after 24 hours of post treatment with quinalophos @ 3 ppm were recorded when compared to the same without exposure to *B. thuringiensis* subsp. *kurstaki*.

KEYWORDS

Bacillus thuringiensis;
Plutella xylostella;
CEH;
MFO.

INTRODUCTION

The extent, to which an insect can metabolise and degrade a toxic chemical, determines its survival in an environment where frequent chemical sprays are given. Induction is a process in which a chemical stimulus enhances the activity of the detoxification systems by the production of additional enzymes. The first report of enzyme induction in insect was made[2], when DDT was applied to *Triatoma infestans* (Klug.) resulted in the increased production of NAD kinase.The three most important systems of detoxification in insects are the microsomal oxidases, the glutathion-s-transferases (GST) (of importance in the metabolism of organophosphorous insecticides or OP) and the carboxyl esterases (which degrade carbamate, OP as well as the juvenile hormone and its analogue)[22]. Higher carboxylesterase activity hydrolysing malathion in resistant *Culex tarsalis* Coquillet. Oppenoorth[15]. *P.xylostella* resistance to synthetic pyrethroids, carbofuran and carbaryl, propoxur and methomyl was due to three separate MFOs[7]. *P.xylostella* was found to posses a natural MFO with metabolic activity towards diflubenzuron and triflumuron[8]. Resistance of *P.xylostella* to pyrethroids, chitin synthesis inhibitors and abamectin were due to induction of MFO[23].

MATERIALS AND METHODS

Preparation of host insects

Enzyme assay was carried out to study the extent of induction of detoxifying enzymes in *P.xylostella* larvae treated with organo phosphorous insecticide quinalphos a '*priori*' and the influence of *B.thuringiensis* infection on the activity level of insecticide detoxifying enzymes namely carboxyl esterase (CE) and mixed function oxidase (MFO). The following treatment combinations were included for the experiment.

I. *B.thuringiensis* + Quinalphos
II. Quinalphos + *B.thuringiensis*
III. *Bacillus thuringiensis*
IV. Quinalphos

Discriminating dose of quinalphos (3 ppm) was prepared using acetone. One ml of the insecticide was pipetted out into a 20 ml glass vial and rolled for 2-3 mts to obtain uniform coating. Upon drying of the insecticide, ten 3rd instar larvae of *P.xylostella* were released in each vial. The mouth of the vial was covered with muslin cloth and fastened with rubber bands. Twelve hours later after exposure to insecticides, the larvae were provided with leaf bits of cauliflower as feed. A strip of filter paper (5 × 0.3 cm) was also kept inside the vial to absorb moisture. In the case of *B.thuringiensis* treatment, the larvae were fed with leaf discs contaminated water with the bacterial spores (LC_{50}). In the case of bacteria + chemical treatment, the larvae were allowed to feed on leaf discs contaminated with bacteria '*a priori'* for 12 hours and then exposed to discriminating dose of quinalphos for another twelve hours. Likewise in the case of chemicals + bacteria treatment, the larvae were exposed to discriminating dose of quinalphos '*a priori*' as described above and subsequently fed with leaf discs contaminated with *B.thuringiensis* (LT_{50}). An untreated check with III instar larvae was maintained as control. The experiment was replicated three times, each with ten larvae. The surviving larvae were used for enzyme analysis.

Protein estimation

The protein estimation was done by following the method[5].

Reagents

1. Stock bradford dye solution

One hundred mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95 per cent ethanol. To this 100 ml of concentrated phosphoric acid was added and finally the volume was made upto 200 ml with distilled water. The dye was stable at 4^0C for six months.

2. Working dye solution

The stock solution was diluted five times with distilled water. The diluted dye solution was prepared just before the assay.

3. Protein standard (Stock)

Bovine serum albumin 50 mg was dissolved in distilled water and the volume was made upto 50 ml.

4. Working standard

Five ml of the stock standard solution was diluted to 50 ml with distilled water to obtain 500 μg of protein ml^{-1} of solution.

Bovine serum albumin standard graph

Protein solutions of concentrations ranging from 20-100μg were prepared by pipetting 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard solution into a series of test tubes. The volume in all the test tubes was made upto 1.0 ml with distilled water. A test tube with 1.0 ml of distilled water served as blank. Five ml of diluted Bradford dye solution was added to each test tube, mixed well and allowed for colour development for at least five minutes but not longer than 30 minutes. The blue colour developed was measured at 595 nm using a spectrophotometer (Spectrophotometer-Perkin Elmer UV/VIS Spectrometer/Lambda Bio). A standard graph was drawn by plotting concentrations of protein along the X-axis and the readings for absorbance along the Y-axis.

Protein estimation in enzyme extract

To one ml of enzyme extract, 5 ml of Bradford reagent was added and allowed for colour development. The absorbance was read at 595 nm. Using the standard graph, the quantity of protein in the enzyme extract was calculated.

Carboxylesterase (CE) assay[9]

Reagents

i. 20 mM phosphate buffer (pH 8.0)
(a).20 mM dibasic sodium phosphate solution: 0.705 g in 250 ml distilled water; (b). 20 mM monobasic sodium phos phate solution: 0.272 g in 100 ml distilled water. 94.7 ml of solution (a) was added to 5.3 ml of solution (b).

ii. Substrate solution
(a). 100 mM stock α-naphthyl acetate solution: 1.862 g dissolved in 100 ml acetone and stored at 0 - 4^0C; (b).1 mM working-naphthyl acetate solution: 1 ml of (a) was made upto 100 ml with 20 mM phosphate buffer (pH 8.0) just before the assay.

iii. Coupling reagent
(a). Fast blue salt solution: 0.3 g in 30 ml distilled water; (b). Sodium lauryl sulphate solution: 3.5 g in 70 ml distilled water, Solutions (a) and (b) were mixed at 2:5 proportion and stored at room temperature.

iv. Standard α-naphthol
(a). 10 mM α-naphthol standard stock solution: 0.144 g

in 100 ml of acetone; (b). 0.1 mM working standard solu tion: 1 ml (a) was made upto 100 ml using 20 mM phos phate buffer (pH 8.0).

α-naphthol standard graph

Different aliquots (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 ml) of -naphthol working standard solution were pipetted out into a series of test tubes. The volume was made upto 5 ml with 20 mM phosphate buffer. The mixture was incubated at room temperature for 30 minutes. The reaction was stopped by addition of 1.0 ml of coupling reagent and the absorbance was measured at 600 nm against a reagent blank in a spectrophotometer. A standard graph was prepared with concentration on X-axis and absorbance on Y-axis.

Enzyme homogenate preparation

The larvae surviving after treatment as described earlier were used for enzyme homogenate preparation. Ten pre-weighed larvae of *P.xylostella* were homogenized in ice-cold 20 mM phosphate buffer (pH 8.0) containing 0.2 per cent triton X-100 using pre-chilled pestle and mortar. Five ml of phosphate buffer was used for extraction. The homogenate was centrifuged at 15,000 rpm for 10 minutes and the supernatant collected served as enzyme source for the assay.

Enzyme assay

Five ml of the working substrate solution was mixed with 1 ml of enzyme homogenate. After 30 minutes of incubation at room temperature, 1 ml of coupling reagent was added. A red colour developed immediately, which changed to fairly stable blue colour, was measured at 600 nm. The specific activity (SA) of the enzyme was estimated using the formula, which was expressed as n moles of α-naphthol released minute^{-1} mg of protein^{-1}.

$$SA = \frac{\mu g \text{ of } \alpha - \text{naphthol released}}{\text{Molecular weight of}} \times \frac{1}{30} \times \frac{1000}{\mu g \text{ of protein}} \times 1000$$

Mixed function oxidases (MFO) assay[11]

Reagents

i. Sucrose medium
(a). 0.24 M sucrose : 41 g in 500 ml water; (b). 1 mM ethylene dinitro tetra acetic acid (EDTA) : 0.186 g in 500 ml water; c. 1 per cent polyvinyl pyrrolidine (PVP) : 1 g in 100 ml water; d. 5 mM phenyl methyl sulfonyl fluoride (PMSF): 0.087 g in 100 ml water
The reagents a, b, c and d were mixed in 2:1:2:1 ratio.

ii. 5 mM Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH): 4.0 mg in 10 ml water

iii. 50 mM tris buffer, pH 7.8 : 3.035 g in 500 ml water (pH adjusted with 0.1 N HCl to 7.8)

iv. 0.01 M p-nitroanisole (PNA) : 30 mg of PNA in 20ml ethanol

v. 1 M sodium hydroxide: 20g in 500 ml water

vi. Standard para-nitrophenol (Stock) : 0.139 g of para-nitrophenol in 100 ml ethanol

vii. Working standard : 1ml of standard stock solution was made upto 10 ml using 50 mM tris buffer of pH 7.8.

Para-nitrophenol standard graph

Different volumes (50, 100, 150, 200 and 250 µl) of para-nitrophenol working standard solution were pipetted out into a series of test tubes. To this 500 µl of tris buffer (pH 7.8) and 20 µl of p-nitroanisole were added. To this 50 µl of NADPH was added in dark and the tubes were allowed to incubate in dark at room temperature for 30 minutes. The reaction was stopped by adding 0.5 ml of sodium hydroxide. The reaction mixture was centrifuged for 10 minutes at 10,000 rpm. The absorbance of the supernatant was measured at 400 nm. A graph was plotted taking concentration on X-axis and absorbance on Y-axis.

Enzyme homogenate preparation

The larvae surviving after the treatment were used for enzyme homogenate preparation. Ten larvae were homogenized in 5 ml of ice-cold sucrose medium using pre-chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 minutes and resultant supernatant was again centrifuged at 16,000 rpm for 30 minutes. The supernatant was used as enzyme source.

Enzyme assay

To 500µl of enzyme source, 500 µl of tris buffer (pH 7.8) and 20 µl of p-nitroanisole were added. To this 50 µl of NADPH was added in dark at room temperature for 30 minutes. The reaction was stopped by adding 0.5 ml of sodium hydroxide. The reaction mixture was centrifuged at 10,000 rpm for 30 minutes. The absorbance of the supernatant was determined at 400 nm. The specific activity (SA) of the enzyme was calculated using the formula, and expressed as n moles of

p-nitrophenol released minute^{-1} mg of protein^{-1}.

$$SA = \frac{\mu g \text{ of } \alpha - \text{nitrophenol released}}{\text{Molecular weight of p - nitrophenol (139.11)}} \times \frac{1}{30} \times \frac{1000}{\mu g \text{ of protein}} \times 1000$$

Statistical analyses

The data on dose mortality responses were subjected to probit analysis[10] after making the necessary corrections[1]. The probit analyses were done using SPSS version 7.1 the ANOVA were done using IRRISTAT.

RESULTS AND DISCUSSION

Activity of carboxylesterhydrolase (CEH) in *P.xylostella*

The data on the activity of carboxylesterhydrolase in *P.xylostella* are presented in TABLE 1. The level of enzyme activity found to be increased in all the treatments as the larvae grew older. It was also found that exposure of *P.xylostella* to *B.thuringiensis* curtailed the synthesis of CEH.

The larval population exposed to quinalphos alone showed CEH activity of 266.60, 297.89, 334.42 and 345.35 n moles min^{-1} of protein^{-1} after 12, 24, 36 and 48 hours of treatment at discriminating dose (3 ppm), respectively, while the control treatment showed CEH activity of 84.34, 173.35, 212.74 and 265.37 n moles min^{-1} mg of protein^{-1}, respectively.

The larval population exposed to *B.thuringiensis* subsp. *kurstaki* after treating with quinalphos showed CEH activity of 259.73, 297.68, 307.37 and 311.88 n moles min^{-1} mg of protein^{-1} after 12, 24, 36 and 48 hours of treatment. The larval population exposed to *B.thuringiensis* subsp *kurstaki a priori* showed CEH activity of 232.26, 280.86, 293.11 and 297.75 n moles min^{-1} mg of protein^{-1} after 12, 24, 36 and 48 hours of treatment with quinalphos @ 3 ppm, respectively, while the untreated check recorded a CEH activity of 84.34, 173.35, 212.74 and 265.37 n moles min^{-1} mg of protein^{-1}, respectively.

The results obtained from the present study on the quantitative assay of carboxylesterhydrolase (CEH) indicated the activity of the enzyme, in general increased as the exposure period of treatment with quinalphos @ 3 ppm increased with or without exposure to *B.thuringiensis* subsp. *kurstaki*. The enzyme activity reduced, when *B. thuringiensis* subsp *kurstaki* applied á *priori* to quinalphos. The findings are in agreement with the result of[20], who opined carboxylesterase hydrolysis for malathion might be a mechanism of organo phosphorus (OP) resistance in *P.xylostella*. The most resistant strain of *H.armigera* (Hubner) to the insecticides, recorded the highest carboxylesterhydrolase activity in the laboratory culture having the least resistance[17]. A significant reduction in the carboxyl esterhydrolase titre in the resistant population of *P. xylostella*, pre-exposed to *B.thuringiensis* subsp. *kurstaki* after 60 hours of post treatment with quinalphos @ 3 ppm, when compared to the same population without exposure to *B. thuringiensis* subsp. *Kurstaki*[3](Figure 1).

The sublethal dose of insecticide might lead to hormoligosis of the insect and increase metabolism[4]. A strong inhibition of carboxylesterase activity after using *B.thuringiensis* at LC_{25} as pretreatment in resistant strains of *P.xylostella* was also reported[25].

TABLE 1: Activity of carboxylesterhydrolase (CEH) in *P.xylostella*

Sl.no	Treatment	CEH activity n moles min^{-1} mg of protein^{-1}			
		12hr	24hr	36hr	48hr
1	Quinalphos	266.60^{a}	297.89^{a}	334.42^{a}	345.35^{a}
2	*B.thuringiensis*	81.31^{e}	175.23^{c}	216.41^{d}	271.37^{d}
3	Quinalphos+ *B.thuringiensis*	259.73^{b}	297.68^{a}	307.37^{b}	311.88^{b}
4	*B.thuringiensis*+ Quinalphos	232.26^{c}	280.86^{b}	293.11^{c}	297.25^{c}
5	Control*	84.34^{d}	173.35^{d}	212.74^{e}	265.37^{e}

***Treated with acetone, Means superscripted with common alphabets are not significantly different at the 5% level by DMRT**

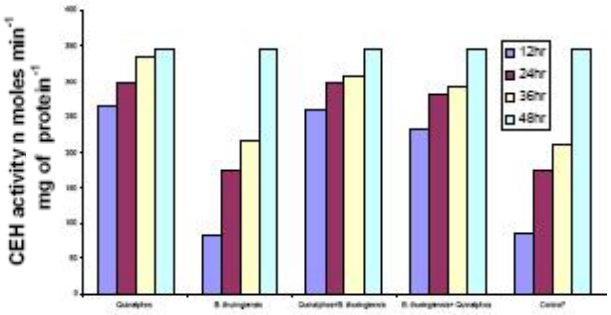

Figure 1: Activity of carboxylesterhydrolase (CEH) in *P. xylostella*

TABLE 2: Activity of mixed function oxidase (MFO) in *P.xylostella*

Sl.no.	Treatment	MFO activity n moles min^{-1} mg of protein^{-1}			
		12hr	24hr	36hr	48hr
1	Quinalphos	16.45^{a}	22.25^{a}	25.42^{a}	29.25^{a}
2	*B.thuringiensis*	14.45^{c}	17.27^{c}	21.48^{b}	25.31^{c}
3	Quinalphos+ *B.thuringiensis*	15.29^{b}	19.38^{b}	25.31^{a}	26.17^{b}
4	*B.thuringiensis*+ Quinalphos	14.21^{d}	16.32^{d}	19.41^{c}	23.16^{d}
5	Control*	14.11^{d}	15.41^{e}	17.38^{d}	19.44^{e}

***Treated with acetone means superscripted with common alphabets are not significantly different at the 5% level by DMRT**

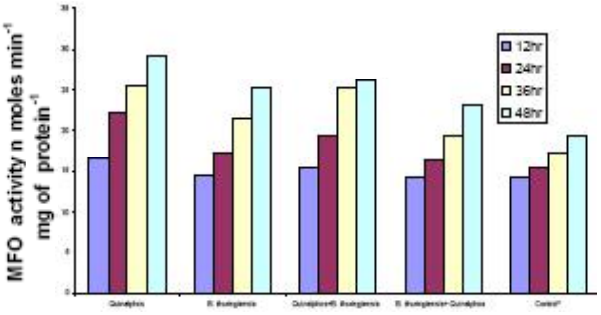

Figure 2: Activity of mixed function oxidase (MFO) in *P. xylostella*

Activity of mixed function oxidase (MFO) in *P.xylostella*

The data on the activity of mixed function oxidase in *P.xylostella* are presented in TABLE 2. The level of enzyme activity found to be increased in all the treatments as the larvae grew older. Further, it is found that exposure of *P.xylostella* to *B.thuringiensis* curtailed the synthesis of MFO. The larval population exposed to quinalphos alone showed MFO activity of 16.45, 22.25, 25.42 and 29.25 n moles min^{-1} mg of protein^{-1} after 12, 24, 36 and 48 hours of treatment at discriminating dose (3 ppm) respectively, while the control treatment showed MFO activity of 14.11, 15.41, 17.38 and 19.44 n moles min^{-1} mg of protein^{-1} respectively.

The larval population exposed to *B.thuringiensis* subsp *kurstaki* alone showed MFO activity of 14.45, 17.27, 21.48 and 25.31 n moles min^{-1} mg of protein^{-1} after 12, 24, 36 and 48 hours of treatment. The larval population exposed to *B.thuringiensis* subsp. *kurstaki* after treating with quinalphos showed MFO activity of 15.29, 19.38, 25.31 and 26.17 n moles min^{-1} mg of protein^{-1} after 12, 24, 36 and 48 hours of treatment. The larval population exposed to *B.thuringiensis* subsp. *kurstaki 'a priori* showed MFO activity of 14.21, 16.32, 19.41 and 23.16 n moles min^{-1} mg of protein^{-1} after 12, 24, 36 and 48 hours of treatment with quinalphos @ 3 ppm respectively, while the untreated check recorded a MFO activity of 14.11, 15.41, 17.38 and 19.44 n moles min^{-1} mg of protein^{-1} respectively.

In the present investigation the level of MFO increased with the age of insects. The activity of MFO found to get reduced in *P.xylostella* larvae exposed to *B. thuringiensis á priori,* whereas a significant induction of MFO was observed in control population. Mixed function oxidases are known to be involved in the oxidation of cyclodiene, synthetic pyrethroids, carbamates, chitin synthesis inhibitors, Avermectin, nereistoxin and to a lower extent organo phosphorus[19,14,26]. Mixed function oxidase were also reported in the degradation of pyrethroids and carbamates in *P.xylostella* change[6,21,26,16,18]. Fat bodies were considered to be the targets for major source of haemolymph protein[24]. Application of microbials like protozoans and *B.thuringiensis* in conjuction with insecticides had been known to break down insecticidal resistance. Increased susceptibility to commonly used insecticides has been reported with application of *B.thuringiensis*[12].

In the present study suppression of both the enzymes CEH and MFO were not drastic, yet a significant reduction was observed. Since *B.thuringiensis* upon infection destroy cell synthesis of proteins such as enzymes CEH and MFO get reduced (Figure 2).

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