RESEARCH ARTICLE ▌

Biochemical Basis of Synergism between Entomopathogens and Insecticide Spinetoram against *Plutella xylostella* (L.)

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ABSTRACT

Increasing incidences of insecticide resistance in diamondback moth *Plutella xylostella* (L.), the most ravaging pest of cruciferous vegetables, have stimulated interest in alternative pest management strategies. In this study, we have developed a spinetoram-resistant population of *P. xylostella* and examined the single and combined toxicity of *Bacillus thuringiensis* subsp. *kurstaki* and *Beauveria bassiana* with spinetoram. Additionally, enzyme activities were tested to study the influence of biopesticides in the mitigation of detoxifying enzymes. *P. xylostella* has developed high level of resistance (174.06-fold) against spinetoram after 16 generations of selection. *B. thuringiensis* subsp. *kurstaki* and *B. bassiana* exhibited high toxicity to spinetoram-resistant population of *P. xylostella*. Synergism was observed between *B. thuringiensis* subsp. *kurstaki* + *spinetoram* and *B. bassiana* + *spinetoram* with co-toxicity factor of 30.23 and 22.51, respectively. The joint application of spinetoram + B. bassiana and spinetoram + B. thuringiensis subsp. kurstaki resulted in suppression of mixed-function oxidase activity by 7.93 to 9.23 per cent and carboxyl-esterase activity by 5.64 to 6.18 per cent. Mixtures of biopesticides with spinetoram exhibited synergistic effects and may aid the design of new combinations of management strategies to delay resistant development in *P. xylostella.*

Keywords: *Plutella xylostella, Spinetoram, Biopesticides, Synergism, Detoxifying enzymes*

INTRODUCTION

The diamondback moth (DBM), *Plutella xylostella* (L.) (*Lepidoptera: Plutellidae*), is a serious pest of cruciferous vegetables, reported in more than 80 countries, and causes severe economic damage worldwide including India. The yield losses and management cost associated with this pest are estimated to be US$ 4-5 billion a year and annual crop loss of US$ 16 million in India (Mohan and Gujar, 2003; Furlong *et al.*, 2013). The use of insecticides remains the primary measure for farmers in India to manage *P. xylostella*. The most commonly used insecticides for the management of *P. xylostella* in India are cypermethrin, fipronil, indoxacarb, spinosad, flubendiamide, chlorantraniliprole, and novaluron. Furthermore, the evolution of resistance to these insecticides, and subsequent spray failures, has been reported for *P. xylostella* (Renuka and Regupathy, 1996; Kishore *et al*., 2014; Ramya *et al*., 2016; Shanmugapriya *et al.*, 2019).

The intensive use of a single insecticide leads to the rapid development of resistance in target pest populations. In the last decade, considerable progress has been made in the assessment of toxicity of combinations of toxins or mixtures against several insect pests. Such mixtures or combinations of insecticides increased toxicity against target pests more than that of a single product (Shabbir *et al.,* 2021). However, this could also affect the non-target beneficial species leading to ecological imbalance. Such ecological damage is expected to be lower when a chemical insecticide is combined with a biopesticide specifically targeting the pest species (Delnat *et al.*, 2019). Pest management using microbial pathogens like virus, bacteria, fungi and nematodes has been recognized as a valuable tool in sustainable crop protection (Bhattacharya *et al.*, 2003). Research on testing combinations of microbial pathogens and insecticides is limited. Few studies have reported a combination of entomogenous fungi viz., *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) increased the toxicity of imidacloprid and oxydemeton methyl against *Spilarctia obliqua* (Walker) (Purwar and Sachan, 2006).

Hence, in the present study, we assess the combined action of *Bacillus thuringiensis* subsp. kurstaki and *B. bassiana* with spinetoram against spinetoram-resistant population of *P. xylostella*. In addition, we have also determined the detoxifying

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enzyme activity of cytochrome P450 monooxygenase (MFO), glutathione S-transferase (GST), and carboxyl-esterase (CarE) to find the mitigation of enzyme induction by joint action of fungi/bacterial pathogens and spinetoram. Thus, determining the joint action of fungi/bacterial pathogens and spinetoram will throw much-needed light on how to improve the management of this pest.

MATERIALS AND METHODS
Insecticide and biopesticide

The insecticide and biopesticides used were spinetoram 11.7 SC, purchased from Dow Agro Sciences India Pvt. Ltd., the commercial formulation of *B. thuringiensis* subsp. *kurstaki* (Delfin®WG) was purchased from Margo Biocontrol Pvt. Ltd., Bangalore, and B. bassiana inoculum was maintained in the Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore.

Bioassay

The concentration-mortality response of spinetoram-resistant population (SPI-R) of *P. xylostella* against *B. thuringiensis* subsp. *kurstaki, B. bassiana* and the mixture of spinetoram + Btk and spinetoram + Bb were conducted with newly emerged third-instar larvae by the standard leaf-dip method (Gao *et al.*, 2018). Cauliflower leaves were cut into spherical discs (6 cm in diameter) and immersed in various test concentrations of insecticide and biopesticides for the 20s, which were prepared with distilled water containing 1 g L^{-1} of Triton X-100. The leaves were allowed to air dry for 1h and then placed individually into a breeding dish (10cm in diameter, 4.0cm in depth) with lightly moistened filter paper. About 10-15 pre-starved (for 2h) third-instar larvae were introduced into each dish along with one control dish that contained distilled water with 1 g L^{-1} of Triton X-100 maintained under laboratory conditions of 25 ± 1°C, 70-90% RH, and a photoperiod of 16L: 8D. Each concentration was replicated thrice, including the control, and mortality was assessed after 48h of exposure. Larvae unable to move when touched with a fine brush were considered to be dead.

Joint action of spinetoram and biopesticides

The joint action of biopesticides and spinetoram was estimated by combining LC_{25} values of spinetoram with LC_{25} values of *B. thuringiensis* subsp. *kurstaki* and *B. bassiana*. The bioassay was performed by leaf disc method and the mortality was observed after 48h of exposure as described above.

The joint action bioassays were accompanied by the spinetoram and the biopesticides alone for better comparison. The value of the co-toxicity factor given by Subbanna *et al.* (2019) was used to describe the joint toxicity of mixtures.

Co-toxicity factor= ((Observed Mortality-Expected Mortality))/(Expected Mortality) x100

A positive co-toxicity factor of +20 or higher means potentiation, a negative factor of −20 or lower means antagonism, and between −20 and+20 means additive interaction.

Enzyme Activity Assay

Enzyme assays were carried out to study the influence of fungal/bacterial infection on the activity levels of insecticide detoxification enzymes (MFO, GST and CarE) in *P. xylostella*. Third instar larvae of *P. xylostella* were allowed to feed on the treated cauliflower leaves. The larvae surviving after treatment were homogenized to extract the enzymes at specific time intervals of 3, 48, and 96 hours after treatment (HAT) (Kranthi, 2005). From the homogenate, the protein was estimated by the method of Bradford (1976). The MFO was assessed according to the method prescribed by Cheng *et al.* (1986), GST activity test was conducted by the method as reported by Habig et al. (1974) and CarE assay was performed by the method as described by Devonshire (1977). For each enzyme assay, four replications were maintained. The per cent increase or decrease in enzymes activity was compared with the control

Statistical Analysis

An Excel workbook was prepared by following the procedure given by Finney (1971) and the mortality data were subjected to Probit analysis at $P > 0.05$. The natural mortality observed in the control was corrected using Abbott (1925) correction. Data obtained from the experiment were analyzed by the analysis of variance (ANOVA) test. The significant difference among treatment groups was analyzed by Tukey's HSD test at $P < 0.05$ (SPSS ver. 21).

RESULTS AND DISCUSSION
Resistance selection of *Plutella xylostella* to spinetoram

The selection process of spinetoram resistance was carried out under laboratory conditions. The LC_{60} concentration of each generation was used for resistance selection in subsequent generation.

After 15 generations of selection, the LC_{50} value of spinetoram against *P. xylostella* has increased significantly from 0.114 mg L^{-1} to 19.843 mg L^{-1} with a resistance ratio of 174.06-fold (Table 1).

Toxicity of biopesticides against spinetoram-resistant population of *Plutella xylostella*

Leaf-dip bioassays revealed that the biopesticides, *B. thuringiensis* subsp. *kurstaki* and *B. bassiana* varied in their toxicity to SPI-R population of *P. xylostella* (Table 2). The LC_{50} value of *B. thuringiensis* subsp. *kurstaki* was 2.08 gL^{-1} with the fiducial limit of 1.74 gL^{-1} to 2.48 gL^{-1} and the LC_{25} value was 1.103 gL^{-1}. Similarly, the LC_{50} value of *B. bassiana* was 2.66 x10$_8$ spores mL^{-1} at the fiducial limit of 1.06 x10$_7$ spores mL^{-1} to 6.60 x10$_8$ spores mL^{-1} and the LC_{25} value was 1.01 x10$_7$ spores mL^{-1}

Joint action of combinations

The combinations of spinetoram with *B. thuringiensis* subsp. kurstaki and *B. bassiana* showed increased toxicity against SPI-R population of *P. xylostella* (Table 2). The mortality of LC_{25} concentration of spinetoram, *B. thuringiensis* subsp. *kurstaki* and *B. bassiana* was found to be 23.33 ± 1.72, 21.11 ± 1.49 and 26.67 ± 2.05 per cent, respectively. The joint action of spinetoram + *B. thuringiensis* subsp. *kurstaki* exhibit higher mortality of 62.22 ± 1.41 per cent over the theoretical mortality of 47.48 per cent. Similarly, spinetoram + *B. bassiana* also exhibited 54.45± 3.18 per cent mortality which was higher than the the or etical mortality of 44.44 per cent (Figure 1). The co-toxicity factor was estimated to find the possible interactions among the two biopesticides. The co-toxicity factor of spinetoram + *B. thuringiensis* subsp. *kurstaki* and spinetoram + *B. bassiana* were 30.23 and 22.51, respectively. Both the biopesticides exhibited a synergistic interaction with spinetoram with the co-toxicity factor values of >20. Similarly, Shabbir *et al*. (2021) found that the combination of *B. thuringiensis* (LC_{50}) + chlorantraniliprole (LC_{50}) was more toxic than the individual treatment, suggesting that synergistic interaction presents between *B. thuringiensis* and chlorantraniliprole. Likewise, synergistic interaction was also observed between *B. thuringiensis* and the insecticides, cypermethrin, profenophos, chlorpyriphos, indoxacarb, and spinosad against *H. armigera* (Duraimurugan and Regupathy, 2004; Subbanna *et al.,* 2019)

Mitigation of detoxifying enzyme activity

The activities of detoxification enzymes were

increased in all the treatments. Application of spinetoram enhanced MFO (154.21 n moles min^{-1} mg protein^{-1}), GST (376.53 n moles min^{-1} mg protein^{-1}) and CarE (694.25 n moles min^{-1} mg protein^{-1}) activities at 3 HAT. However, the enzyme activity was minimum in *B. bassiana* and *B. thuringiensis* subsp. *kurstaki* treated larvae at 3 HAT and it was enhanced at 48 HAT. At 6 HAT the activity of MFO was decreased in spinetoram + *B. bassiana* (0.24 per cent) treatment while it was slightly enhanced in spinetoram + *B. thuringiensis* subsp. *kurstaki* (0.68 per cent) over the spinetoram treatment alone. The MFO activity increased in all the treatments as the larvae developed. However, the levels were significantly lower than the spinetoram treatment alone. The joint application of spinetoram + *B. bassiana* and spinetoram + *B. thuringiensis* subsp. *kurstaki* resulted in suppression of MFO activity to the extent of 7.93 to 9.23 per cent over the spinetoram treatment alone. A similar trend was also observed in the case of CarE activity. The extent of CarE suppression in joint application of spinetoram + *B. bassiana* and spinetoram + *B. thuringiensis* subsp. kurstaki was 5.64 to 6.18 per cent over the spinetoram treatment alone. The activity of GST was slightly enhanced by the spinetoram treatment over control. Since the reduced activity of GST, the enzyme suppression was also minimum in the joint application of spinetoram + *B. bassiana* (0.18 to 1.91 per cent) and spinetoram + *B. thuringiensis* (0.16 to 2.65 per cent). Similarly, Adhira et al. (2004) also observed strong inhibition of CarE and GST activity after joint application of *B. thuringiensis* with quinalphos in *P. xylostella*. Ali *et al*. (2017) also reported significant suppression of CarE and GST activity after the combined application of chemical insecticide and *L. muscarium* in *B. tabaci*.
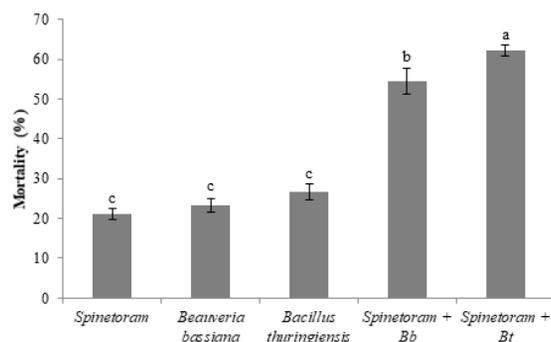
Figure 1. Joint action of *Beauveria bassiana* (LC_{25}) and *Bacillus thuringiensis* (LC_{25}) with spinetoram (LC_{25}) against spinetoram-resistant *Plutella xylostella*

Table 1. Spinetoram resistance changes in the laboratory population of *Plutella xylostella* with continuing selection

Generation	n	Slope	χ^2	df	LC$_{50}$ (mg L^{-1}) (95% FL)	RR
F$_0$	360	2.813	1.680	5	0.114 (0.097 - 0.133)	-
F$_5$	360	1.970	2.466	5	0.301 (0.253 - 0.359)	2.64
F$_{10}$	360	4.717	1.447	5	5.702 (5.305 - 6.128)	50.02
F$_{15}$	360	3.126	1.807	5	19.843 (17.688 - 22.260)	174.06

n - Total number of larvae exposed to bioassay experiment

RR - Resistance ratio calculated as: RR=LC$_{50}$ of F$_n$ / LC$_{50}$ of F$_0$

Table 2. Toxicity of *Beauveria bassiana* and *Bacillus thuringiensis* subsp. *kurstaki* against spinetoram-resistant *Plutella xylostella*

Beauveria bassiana						
n	Slope ± SE	χ^2	LC$_{25}$ (x10$_7$ spores mL^{-1})	Fiducial limit (x10$_6$ to 10$_8$ spores mL^{-1})	LC$_{50}$ (x10$_8$ spores mL^{-1})	Fiducial limit (x10$_7$ to 10$_8$ spores mL^{-1})
270	0.498	1.616	1.010	2.27 - 4.48	2.658	1.06 - 6.60
Bacillus thuringiensis subsp. kurstaki						
n	Slope ± SE	χ^2	LC$_{25}$ (gL^{-1})	Fiducial limit (gL^{-1})	LC$_{50}$ (gL^{-1})	Fiducial limit (gL^{-1})
270	2.514	2.198	1.103	0.811 - 1.496	2.075	1.736 - 2.480

n - Total number of larvae exposed to bioassay experiment

Table 3. Mitigation of MFO, GST and CarE induction due to spinetoram in *Plutella xylostella* by *Beauveria bassiana* and *Bacillus thuringiensis* subsp. *kurstaki*

HAT	Mixed function oxidase					Increase(+) / Decrease (-) over resistant stain	
	Spinetoram	B. Bassiana	B. thuringiensis	Bb + Spinetoram	Bt + Spinetoram	Bb + Spinetoram	Bt + Spinetoram
6	154.21c	147.84a	151.04b	154.58c	155.26c	-0.24	0.68
24	179.35d	152.13a	156.55b	176.03c	174.67c	-1.85	-2.91
48	196.90d	155.78a	161.19b	181.28c	178.73c	-7.93	-9.23
Control	147.73	-	-	-	-	-	-
	Glutathione S-transferase						
6	376.53b	369.37a	370.07a	377.22b	376.73b	-0.18	0.05
24	383.40c	372.88a	376.01b	385.70d	382.78c	0.60	-0.16
48	398.76d	381.48a	382.75a	391.14c	388.21b	-1.91	-2.65
Control	368.54	-	-	-	-	-	-
	Carboxyl esterase						
6	694.25b	659.76a	660.13a	695.08b	695.46b	-0.12	0.17
24	722.11e	664.13a	667.36b	704.54d	701.72c	-2.43	-2.82
48	753.38e	678.37a	681.21b	710.91d	706..84c	-5.64	-6.18
Control	658.76	-	-	-	-	-	-

HAT indicates hours after treatments

Enzyme activity in n moles min^{-1} mg protein^{-1}

Mean of four replications

CONCLUSION

The significant synergistic effects were observed from mixtures of biopesticides with spinetoram against insecticide-resistant population of *P. xylostella* suggests a practical approach that can delay or overcome common pesticide resistance mechanisms. The combination mixtures retained a high level of toxicity than the individual treatment. These results support the idea of the potential use of biopesticides with insecticides in ways that will considerably delay resistance in *P. xylostella*.

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All the authors agreed to publish the content.

Competing Interests

The authors declare no conflict of interest in publication of this content

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