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RESEARCH PAPER

Assessment of non-target effects of Bt proteins (Dipel 8L, Vip3Aa70, and Vip3Aa71) on the developmental biology of *Bracon hebetor* Say parasitizing *Chilo partellus* (Swinhoe)

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Abstract: *Bacillus thuringiensis* (Bt) based bioinsecticides and Bt proteins are widely used for the management of lepidopteran pests. However, their potential effects on non-target beneficial insects such as parasitoids require careful evaluation. The present study assessed the impact of Bt formulations and Vip proteins on the parasitoid *Bracon hebetor* through its host, the maize stem borer, *Chilo partellus.* Second instar larvae of the maize stem borer were exposed to LC_{50} concentrations of Dipel 8L and Vip proteins (Vip3Aa70 and Vip3Aa71) for 48 hours. The LC_{50} values were 1.45 ppm for Dipel 8L, 4.17 ppm for Vip3Aa70 and 4.83 ppm for Vip3Aa71. After feeding on Bt proteins at LC_{50}, third-instar *C. partellus* larvae were exposed to *Bracon hebetor* and the developmental parameters of the parasitoid were recorded. Cocoon period (days), cocoon weight (mg) and cocoon size (mm) of the parasitoid showed non-significant differences among treatments, indicating only mild effects on the biological traits. Cocoon formation (%) and adult emergence (%) differed significantly; Dipel 8L resulted in the lowest adult emergence, while cocoon formation was minimum in the Vip protein (Vip3Aa70 and Vip3Aa71) treatments, though differences among Vip proteins were non-significant. The exposure at LC_{50} concentrations of Dipel 8L and Vip3A proteins caused slight reductions (5.95-7.59 % in cocoon formation and 6.86-10.33% in adult emergence) and thus exerted no major adverse effects on the developmental biology of *B. hebetor*.

Keywords: *Bracon hebetor* • *Chilo partellus* • Dipel 8L • Non-target toxicity • Vip proteins

Introduction

Maize (*Zea mays* L.) is a major global cereal crop, with India ranking fifth in production (FAOSTAT, 2024). Maize productivity is severely affected by the spotted stem borer, *Chilo partellus*, causing yield losses caused by *C. partellus* vary widely depending on agro-climatic conditions, infestation levels and crop stage, ranging from 26.7 to 80.4% across different agro-climatic zones of India (Chatterji *et al.*, 1969) and average avoidable losses caused by *C. partellus* were found to be 12.99 per cent (Dhaliwal *et al.,* 2018). *Bacillus thuringiensis* (Bt) proteins offer an effective alternative to chemical control and Cry proteins are well established for managing lepidopteran pests (Singh *et al.*, 2005). Vegetative insecticidal proteins (Vip3A) proteins are promising due to their distinct mode of action, low cross-resistance risk, narrow host range and compatibility in transgenic crops (Chakroun *et al.*, 2016; Gupta *et al.*, 2021). Although Bt proteins are target-specific and generally safe for beneficial arthropods (Naranjo, 2005; Raybould and Vlachos, 2011) but assessing their non-target effects is essential in different agro ecological zones. *Bracon hebetor*, a gregarious idiobiont ectoparasitoid wasp of *C. partellus*, that parasitizes the larvae of several other lepidopteran species and is widely recognized as an effective biological control agent. Females of *B. hebetor* paralyze the host with venom

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and lay eggs on or near it; the larvae feed on the host, kill it, and finally leave to form white cocoons for pupation (Dabhi *et al.*, 2011; Tuteja and Shera, 2025). Therefore present study evaluated the non-target toxicity of Dipel 8L and Vip proteins (Vip3Aa70 and Vip3Aa71) on *B. hebetor* under laboratory conditions focusing on survival, parasitization and adult emergence following exposure to treated larvae of *C. partellus* at LC_{50} concentrations.

Materials and methods

Experimental materials

The Bt formulation Dipel 8L (*Bacillus thuringiensis* var. *kurstaki*, strain HD-1, serotype 3a, 3b; 3.5%) was procured from Sumitomo Chemicals India Pvt. Ltd. Crude Vip3Aa proteins (Vip3Aa70 and Vip3Aa71) were obtained from the Molecular Biology and Biotechnology Laboratory, ICAR–Indian Institute of Maize Research (IIMR), Ludhiana. These proteins were expressed from recombinant *Escherichia coli* BL21 (DE3) cells containing *vip3Aa* genes cloned into pET29a(+) expression vector, which were isolated from Indian native *Bt* isolates SK-851 and SK-306 and their expression was confirmed by SDS-PAGE and immunostrip assay (Gupta *et al.*, 2024). The stability of the protein was ensured by using a protease inhibitor during extraction to prevent protein degradation.

Insect culture

Rearing of Chilo partellus

Larvae of *C. partellus* were collected from maize fields of Ludhiana, Punjab, India and reared on fresh maize whorl pieces in plastic jars (10 × 15 cm) under controlled laboratory conditions (25 ± 1°C, 70 ± 5% RH). For mass rearing, larvae were maintained on a green gram (*Vigna radiata*) based artificial diet prepared following Kanta and Sajjan (1992). Pupae were placed in moist, sand-lined jars until adult emergence. Emerged adults were subsequently transferred to oviposition jars for egg collection as per (Panwar, 2005).

Rearing of Bracon hebetor

The parasitoid *B. hebetor* Say was maintained on late instar larvae of *Corcyra cephalonica* under laboratory conditions (25 ± 1°C, 70 ± 5% RH). Adult parasitoids were provided with 10% honey solution as a food source.

Assessment of sub-lethal concentrations through larval bioassays

The diet incorporation method was followed (Dulmage *et al.*, 1971). Freshly prepared proteins were incorporated into the artificial diet before each bioassay to maintain protein activity during the exposure period. Dose–mortality bioassays were performed using second instar larvae of *C. partellus* on treated artificial diet to determine the LC_{50} values of Dipel 8L and Vip3Aa proteins. The bioassay was performed at seven concentrations (35, 8.7, 2.2, 0.55, 0.14, 0.034 and 0.008 ppm) of Dipel 8L and eight concentrations (50, 25, 12.5, 6.25, 3.125, 1.5, 0.7 and 0.3 ppm) of Vip3Aa proteins (Vip3Aa70 and Vip3Aa71).

Effect of Bt proteins treated third instar Chilo partellus larvae on biological parameters of Bracon hebetor

Second instar larvae of *C. partellus* were exposed to LC_{50} concentrations of Dipel 8L and Vip3Aa proteins for 48 hours. After exposure, larvae were transferred to untreated artificial diet and reared until they reached the third instar. These treated third instar larvae were then offered to *B. hebetor* for parasitization. Ten larvae were exposed to five pairs of adult *B. hebetor* in 1000 mL glass jars for 48 hours using sandwich method (Sree

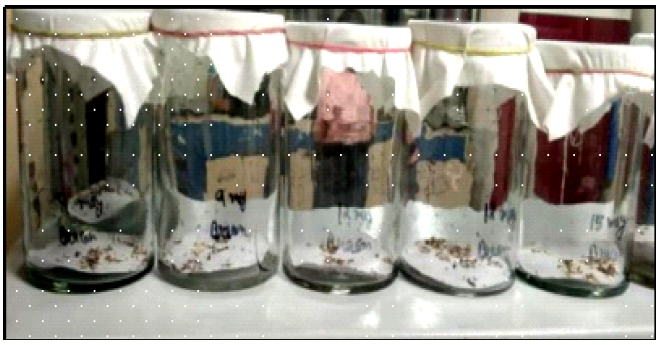

Plate 1. *Chilo Partellus* exposed to *Bracon hebetor*

Plate 2. Parasitization of *Chilo partellus*

latha *et al.,* 2019). Each treatment consisted of eight replications with ten larvae per replication along with the control. All experiments were conducted under controlled laboratory conditions (25 ± 1°C, 70 ± 5% RH). Parasitized larvae were observed daily until cocoon formation and adult emergence. Data were recorded on cocoon formation, cocoon size, cocoon weight and percent adult emergence.

Assessment of developmental parameters of Bracon hebetor

Cocoon formation (%)

Cocoon formation was observed regularly to monitor the progress of cocoon formation in the treated as well as in control larvae. In each replication the total number of cocoons formed was carefully counted until no further cocoon formation was observed. The percentage of cocoon formation was then calculated separately for each protein to assess the effect of treatments on pupation.

$$\text{Cocoon formation (\%)} = \frac{\text{Number of cocoon formed}}{\text{Total number of larvae}} \times 100$$

Cocoon period (days)

The cocoon period was recorded as the number of days from cocoon formation to adult emergence.

Cocoon size (mm)

Cocoon size measurements were taken under a MICRODIRECT 1080p HD handheld digital microscope (Celestron Acquisition, LLC, China) fitted with a calibrated scale. Length of the cocoons were recorded in each replication of test protein. Mean cocoon size was calculated for each treatment and compared with the control.

Cocoon weight (mg)

Cocoon weight of individual cocoons was recorded. Freshly formed cocoons from each replication were carefully collected and weighed using an analytical electric weighing balance (A & D company Limited, Japan).

Adult emergence (%)

Number of adults that successfully emerged from the total cocoons in each of the replicates were recorded for each concentration. This percentage was calculated using the given formula:

$$\text{Adult emergence (\%)} = \frac{\text{Number of adult emerged}}{\text{Total number of cocoon}} \times 100$$

Statistical analysis

The mortality data was subjected to probit analysis (Finney, 1971) using the software POLO-PC (LeOra Software, 1987) to determine the LC_{50} values. The data was analyzed using one-way analysis of variance (ANOVA) and treatment means were compared using least significant difference at 5 per cent level of significance (Gomez and Gomez, 1984).

Results and discussion

Toxicity of Dipel 8L and Vip3Aa proteins against second instar Chilo partellus larvae

The bioassay revealed that in Dipel 8L, after 72 hours, larval mortality ranged from 94.48 per cent at the highest concentration of 35 ppm to 3.50 per cent at the lowest concentration of 0.008 ppm, indicating a clear dose-dependent response. Similarly, Vip3Aa70 protein caused mortality ranging from 89.80 per cent at the highest concentration of 50 ppm to 7.14 per cent at the lowest concentration of 0.3 ppm. For Vip3Aa71 protein, larval mortality varied from 92.78 per cent at the highest concentration of 50 ppm to 3.09 per cent at the lowest concentration of 0.3 ppm. The LC_{50} value of Dipel 8L against second instar larvae of *Chilo partellus* was 1.45 ppm with fiducial limits of 1.28–1.53 ppm. In comparison, Vip3Aa70 and Vip3Aa71 showed LC_{50} values of 4.17 ppm and 4.83 ppm with fiducial limits of 2.29–6.89 ppm and 2.50–9.24 ppm, respectively (Table 1). These LC_{50} values were subsequently used to assess the sub-lethal effects of Bt treatments on parasitization efficiency and developmental parameters of *Bracon hebetor.*

Table 1. Determination of toxicity to *C. partellus* 2nd instar larvae against Dipel 8L and Vip3Aa proteins

	LC_{50} (ppm)	Fiducial ranges
Dipel 8L	1.45	1.28 - 1.53
Vip3Aa70	4.17	2.29 - 6.89
Vip3Aa71	4.83	2.50 - 9.24

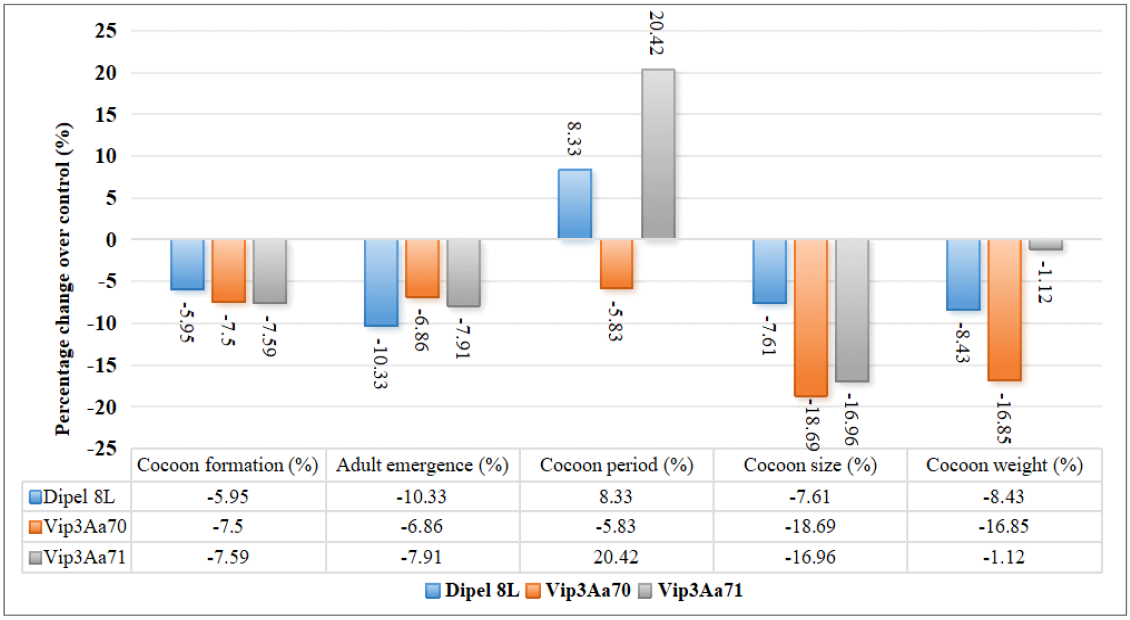

Figure 1. Percentage change in developmental parameters of *Bracon hebetor* exposed to Bt proteins at LC_{50} concentration

Effect of Bt protein treated Chilo partellus larvae on developmental biology of Bracon hebetor

Cocoon formation (%)

The highest cocoon formation of *B. hebetor* was recorded in the control larvae of *C. partellus* (80.65 ± 0.87%), followed by Dipel 8L (75.85 ± 0.99%), Vip3Aa70 (74.60 ± 0.94%) and Vip3Aa71 (74.53 ± 1.46%). All the treatments were significant with each other. Larval development was comparable across treatments with earlier studies (Sedaratian *et al.*, 2014) reporting a slight reduction in parasitoid success and shortened development when hosts were Bt-fed, while Vip3Aa exposure (Ali *et al.*, 2021) caused only mild developmental delays without affecting survival or adult fitness. Although cocoon formation in all treatments was slightly reduced (5.95-7.59%) compared to the control, the differences among Dipel 8L and the Vip proteins were statistically non-significant (Figure 1).

Cocoon period (days)

Cocoon period in the control group, was 4.80 ± 0.82 days. It was 5.20 ± 0.29 days in the Dipel 8L treatment, 4.52 ± 0.86 days in the Vip3Aa70 protein treatment and 5.78 ± 0.74 days in the Vip3Aa71 protein as presented in table 2. All the treatments were non- significant as also reported by Kalyebi *et al.* (2013), the pupal duration of *Habrobracon hebetor* parasitizing *H. armigera* did not differ significantly across sub-lethal concentrations of *Bacillus thuringiensis* ($P > 0.05$), indicating no adverse effect of Bt on parasitoid pupal development. Muslim *et al.* (2018) reported that the cocoon period of *B. hebetor* showed only minor variation across different insecticide treatments with no statistically significant effect on pupal duration ($P > 0.05$).

Cocoon size (mm)

The cocoon size was slightly larger in the control (2.89 ± 0.89 mm) than Dipel 8L (2.67 ± 0.76 mm). However, it was slightly smaller in the Vip3Aa70 (2.35 ± 0.34 mm) and Vip3Aa71 (2.40 ± 0.62 mm) protein treatments (Table 2) and there was no statistically significant difference among the treatments.

Cocoon weight (mg)

Similarly, the cocoon weight was slightly more in the control (1.78 ± 0.63 mg), followed by Dipel 8L (1.63 ± 0.34 mg). However, comparatively lower values were recorded in Vip3Aa70 (1.48± 0.59 mg) and Vip3Aa71 (1.76 ± 1.09 mg) as presented in table 2. Although slight variations were observed in cocoon weight among the treatments but the differences were statistically non-significant aligning with Wang *et al* (2020) who observed no adverse effects of Cry2Aa protein on pupal weight of *H. hebetor.*

Table 2. Effect of Bt proteins at LC_{50} concentrations on the developmental biology of *B. hebetor* parasitizing *C. partellus* larvae

Concentration	Development parameters of *B. hebetor**				
LC_{50} (ppm)	Cocoon formation (%) (Mean ± SE)	Cocoon period (days) (Mean ± SE)	Cocoon size (mm) (Mean ± SE)	Cocoon weight (mg) (Mean ± SE)	Adult emergence (%) (Mean ± SE)
Dipel 8L (1.45)	75.85 ± 0.99[b]	5.20 ± 0.29	2.67 ± 0.76	1.63 ± 0.34	76.43 ± 1.35[c]
Vip3Aa70 (4.17)	74.60 ± 0.94[c]	4.52 ± 0.86	2.35 ± 0.34	1.48 ± 0.59	79.39 ± 0.53[b]
Vip3Aa71 (4.83)	74.53 ± 1.46[c]	5.78 ± 0.74	2.40 ± 0.62	1.76 ± 1.09	78.50 ±0.43G[bc]
Control	80.65 ± 0.87[a]	4.80 ± 0.82	2.89 ± 0.89	1.78 ± 0.63	85.24 ± 0.72[a]
CD (p = 0.05)	1.08	NS	NS	NS	2.87

*Based on 10 larvae/replication (8 replications)

Adult emergence (%)

Adult emergence of *B. hebetor* differed significantly between the control and all other treatments. The highest adult emergence was recorded in the control (85.24 ± 0.72%), whereas the lowest was observed in Dipel 8L (76.43 ± 1.35%). In contrast, emergence rates were slightly higher in the Vip protein treatments, with 79.39 ± 0.53% in Vip3Aa70 and 78.50 ± 0.43% in Vip3Aa71. Overall, these treatments showed only a small reduction (6.86-10.33%) compared to the control, as shown in Figure 1.

Overall, Vip3A proteins (Vip3Aa70 and Vip3Aa71) and Dipel 8L showed minimal non-target effects on *Bracon hebetor.* While slight reductions in cocoon formation and adult emergence were observed, cocoon size, weight and development period remained unaffected. Dipel 8L exhibited marginally higher toxicity than Vip proteins but overall effects were mild, indicating that Vip3A proteins are safe for *B. hebetor* and compatible with integrated pest management programs. Akinkurolere *et al.* (2009) reported that Bt formulations exert sub-lethal effects on *H. hebetor*, reducing oviposition and adult emergence; however, parasitoid development was not completely inhibited, indicating that successful parasitization can still occur on Bt-treated hosts. In the present study, Bt proteins (at LC_{50}) showed the least non-target toxicity to the larval parasitoid *B. hebetor*. Similarly Wang *et al.* (2020), found Cry2Aa remained bioactive in parasitized hosts but had no negative impact on parasitoid development, longevity, or fecundity. Raybould and Vlachos (2011) reported Vip3A protein in MIR162 maize is highly specific to lepidopteran pests. Among 12 tested species, 11 displayed no adverse reactions when exposed to high concentrations of Vip3A. Even under high exposure levels, non-target organisms remained unaffected. These findings support the conclusion that Vip3A has minimal ecological risk and is safe for non-target beneficial organisms. Naranjo (2009) strongly emphasized that Bt crops producing Cry and Vip proteins pose negligible risk to non-target invertebrates due to their high specificity. Abbas (2020) reviewed that the effects of *Bacillus thuringiensis* on natural enemies are variable, with outcomes largely dependent on Bt concentration and timing of host exposure, though most studies report minimal adverse effects on parasitoids and predators.

Conclusion

Exposure of third instar *Chilo partellus* larvae to LC_{50} concentrations of Dipel 8L and Vip3Aa proteins slightly influenced the developmental parameters of the parasitoid, *Bracon hebetor*. Compared to the control, treated larvae showed variations in cocoon formation, cocoon size, cocoon weight and adult emergence of *B. hebetor*. Overall, cocoon formation and adult emergence were moderately reduced in Bt-treated groups, whereas cocoon size and weight showed minor variations compared to the control. These findings indicate that Vip3A proteins and the Bt formulation Dipel 8L are largely compatible with *B. hebetor* under laboratory conditions. The results indicate that Cry and Vip based Bt products may be integrated into maize integrated pest management (IPM) programs with minimal adverse effects on this beneficial parasitoid. However, further studies under semi-field and field conditions are required to confirm these observations before broader ecological implications can be drawn.

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