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Shelf Life and Field Evaluation of CAMB *Bacillus thuringiensis* Biopesticide Against *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) on Tomato

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Abstract: Previous lab report (Karim *et al*., 1999) had shown the efficacy of CAMB *Bacillus thuringiensis* preparation in potato field to control *Helicoverpa armigera*. In present study, CAMB Bt formulations based on locally isolated Bt strain were checked against 1st to 4th instar larvae of *H. armigera* by incorporating Bt proteins into artificial diet. Early instars showed significant susceptibility to Bt formulation. Laboratory assays of stored CAMB Bt formulations did not show any significant change in their toxicity towards *H. armigera* larvae in laboratory assays. Potency of stored Bt formulations for 12 and 24 months were also tested in small scale experiment conducted under contained conditions on tomato crop. All formulations were found effective to control *H. armigera* larvae in tomato crop. The present study shows that CAMB Bt formulation can be used in field applications to control target pests after long storage time.

Key words: *Bacillus thuringiensis*, *Helicoverpa armigera*, Shelf life, Biopesticide

Introduction

Bacillus thuringiensis is a widely distributed, aerobic, rod-shaped gram positive bacterium that produces crystalline protein inclusions known as δ-endotoxins. These are the vanguard of active ingredients for biological control of agronomically important insect pests, household pests, as well as vectors of animal and human diseases (Schnepf *et al.,* 1998). Bt δ-endotoxin has been used successfully as a natural pesticide in agriculture, forestry and public health for several decades due to the emergence of pesticide resistance, resurgence of pests, residues in foodstuff, impaired farmer health, lack of selectivity towards beneficial insects, and environmental down sides from the wide spread use of pesticides (Karim *et al*., 1999). In recent years, information has been obtained on the mode of action of Bt toxins. Mode of action of δ-endotoxins can be described by the following steps; ingestion, solubilization, proteolytic activation, penetration of peritrophic membrane, receptor binding (reversible and irreversible), membrane insertion, ion channel formation and cell lysis as reviewed by Karim and Riazuddin (1997).

Helicoverpa armigera (Hübner) is one of the most devastating pests because it has developed resistance to synthetic pesticides (Karim, 2000). Concern about environment pollution, resistance to pesticides, residues in food and biodiversity make new and novel strategies to combat pests like *H. armigera*, critically important to secure food for a rapidly growing population. In view of these considerations, Bt biopesticide offers a technically feasible and environmentally acceptable strategy for controlling agronomically important insects. The use of Bt based biopesticide in most developing countries is extremely limited for a variety of reasons most notable among them is poor efficacy of imported products under local conditions (Prior, 1989). We have made extensive studies on efficacy, resistance build up and economics of commercial productions (Karim *et al*., 1999a). The present paper reports our findings on shelf life and efficacy against *Helicoverpa armigera* both in lab and small scale tomato field trials. These studies are a continuation of earlier published work (Karim *et al.,* 1999b).

Materials and Methods

Insects: The *H. armigera* colony has been maintained on a diet (Makhdoom *et al.,* 1997) for the last many years. *H. armigera* culture was constantly supplied with moths from the field populations to prevent inbreeding and subsequent loss of vigor. The culture was maintained under conditions of ambient temperature, humidity and light.

Bacterial strain: Locally isolated and characterized CAMB Bt strain highly efficacious against *Helicoverpa armigera*, *Earias vitella*, *Pectinophora gossypiella, Scirpophaga incertulas, Cnaphalocrocis medinalis* (Khan *et al*., 1995; Karim *et al*., 1999b; Makhdoom *et al*., 1997; Khan *et al*., 1995a) was acquired from CAMB culture collection. A commercial formulation "Agree" was generously provided by Novartis, Pakistan.

SDS-PAGE analysis: SDS-PAGE was carried out by the method reported by Laemmli (1970). Bt pesticidal proteins were run on 10% Polyacrylamide gel and stained with Coomassie brilliant blue stain (0.25% Coomassie brilliant blue, 45.5% methanol & 9% Glacial acetic acid) at 65°C and destained at the same temperature.

Biotoxicity assays: The bioassays were carried out on different instars (1st to 4th instar) of *H. armigera* larvae for susceptibility studies and 2nd instar for stability studies on artificial diet . A total of 1 ml of formulation mixed in 40 g of diet and air dried. One larvae per vial (2 g diet each) was placed because of *H. armigera* cannibalistic behavior. The mortality rates were recorded after 3 days and LC_{50} value were calculated through Probit analysis using computer programme Quantal software (Le Ora Software, 1987). At least five concentrations were used to estimate LC_{50} value. Each set of experiment was repeated at least 3 times.

Fermentation and formulation: For the production of Bt biopesticide formulation, the CAMB Bt strain (CAMB Catalogue number, 3-023) was grown in a 14 liter "Microferm fermentor" New Brunswick, USA, model MF-114 using CSL-salts medium for 48 hrs (Zafar *et al*., 1999). The cells were harvested by centrifugation at 7K for 15 minutes in Beckman centrifuge. Biopesticide formulation was prepared in fine powdered form. CAMB1-97 (1% active ingredient), CAMB2-97 (2% active ingredient) and CAMB4-97 (4% active ingredient) formulations were prepared in 1997 and kept at room temperature in the laboratory. CAMB1-98 (1% active ingredient), CAMB2-98 (2% active ingredient) and CAMB4-98 (4% active ingredient) formulations were also prepared in 1998 and stored in lab for future testing.

Shelf life studies: Bt biopesticide formulation prepared in 1997 and 1998 were stored at room temperature in light protected air tight containers. Efficacy of stored Bt formulations was carried out both in lab assays and field trials on tomato field infested with *H. armigera* larvae.

Protein estimation: The crystal protein concentration was

estimated after solubilizing a known quantity of dried cell mass in alkalic buffer (50 mM sodium carbonate, 10 mM dithiothreitol, pH 10) and incubated at 37°C for 4 hrs. Protein concentration of the protoxin was determined by the Bradford method (Bradford, 1976).

Field studies: Studies were conducted in April-May 1999 in the campus of National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. During this study in 1999, the weather was dry and hot with temperature highs ranging from 35-40°C and lows ranging from 24-28°C. No rainfall occurred during the period of application of Bt. These tests were designed to investigate the efficacy, shelf life and eventually field activity of stored Bt formulations against *H. armigera*. Tomato (*Lycopersicon esculentum*), variety "money maker" was transplanted in a tunnel covered structure under contained environment for two months before the 1st spray application.

An area of 196 feet in length and 12 feet in width was divided into 8 plots. Each plot had 4 rows that were 22 feet long and 12 feet wide. Tomato nursery was transplanted in the month of February, 1999. To augment natural populations of *H. armigera*, 1000 laboratory reared pupae were released into tomato plots 3 weeks before the first application. In addition 1000-2000 larvae were infested for population build up in the experimental plots. A pretreatment sampling was carried out on April 30, 1999 in morning hours to determine the infestation level of *Helicoverpa armigera* in tomato plots. A total of 5 plants were selected randomly in each plot to count the number of larvae from top of plant to down including fruits. Each plant was infested with an average of 9 larvae per tomato plant in pretreatment sampling. First treatment of plots with *Bacillus thuringiensis* based formulations were carried on the same day in evening hours. Treatments were applied in a completely random design (Cochran and Cox, 1950). One plot was treated with only water and another plot with Bt formulation. A total of 50 g each of the formulation was stirred in water taken from CAMB water tank and sprayed at a rate of 10 liter per plot with a pressure pump. A total of 2 spray applications were carried out on weekly basis for asynchronous populations. In the third week, plots were left without any spray to augment a synchronous population and then on next week, a final spray application was made. The efficacy of the selected treatments was determined by examining and recording the number of young larvae and older larvae and eggs per five plants per plot before any spray application.

Statistical analysis: Percent efficacy was calculated as reported by Henderson and Tilton (1955). All the data recorded were subjected to analysis of variance using IRRI. (1997) and t-test was applied to determine the significance of difference between values.

Results

Potency of Bt formulation against different instar larvae of the target insect was carried out. LC_{50} values are compared in Table 1. All the LC_{50} values were expressed in µg/g of diet, enabling comparison between the relative toxicities of each instar. All 130 KDa proteins are giving stable proteins. Early instars were significantly more susceptible to Bt toxin than later instars; which were tolerant. First three instars were most susceptible stages tested. Results in Table 1 showed that first instar larvae of *H. armigera* in lab assays were highly susceptible to local Bt formulations. There is a significant difference between the pesticidal efficacy of local Bt formulation and Agree of Novartis (F, 0.05 2.131 > Prob 0.2505) while LC_{50} comparison represents local Bt formulation being more toxic than Agree (Table 2).

Bt formulations were found stable and active during laboratory storage upto 36 months (Table 3). Toxicity after 6-36 months of lab storage reduced by only 6-10% against *H. armigera* (Table 3). LC_{50} values of 0-24 month old Bt formulations were significantly same in our lab assays against 2nd instar larvae of *H. armigera* (Table 4).

Table 1: Efficacy of Bt biopesticide on different growth stages of *H. armigera*

Larval growth stage (Instars)	Bt local formulation, LC_{50} (µg/g of diet)	Agree(Novartis) LC_{50} (µg/g of diet)
1st Instar	15 ± 3.1	23.7 ± 3.8
2nd Instar	80.5 ± 2.5	79 ± 2.5
3rd Instar	75.5 ± 2.9	85.3 ± 1.4
4th Instar	>100	>100

During small scale field studies, the weather was dry, bright sunshine with temperature highs ranging from 35-40°C and lows from 24-28°C. No rainfall or wind blew during the spray applications. Insect populations build up of *H. armigera* on tomato crop was significantly high (Table 5). Average numbers of larvae recorded in pre-treatment plots were 9 larvae per plant. After first spray, a few larvae were found in the fruit plots sprayed with Bt in asynchronous populations of *H. armigera*. No significant difference was found in 1-2 years stored products in experimental plots. After 2nd spray, a complete eradication of *H. armigera* larvae was found in the treated plots. At this stage, we assumed that all the larvae residing in the fruits had either pupated or reached at pupation stage. Therefore, all plots were left untreated and given ample opportunity to all pupated populations to develop into moths for the next generation. In all the plots, eggs and early instar *H. armigera* larvae were recorded (Table 6, 7). Therefore, a third application was applied on the plots. Fewer small larvae were found on the leaves of tomato. After 4th spray, the whole population of *H. armigera* in tomato crop was controlled.

Discussion

Bacillus thuringiensis appears to have great potential for the control of *Helicoverpa armigera*. CAMB Bt formulation was found the most potent formulation against different larval instars tested and as such may be considered the best candidate for field evaluation. It must be borne in mind, though, only early instars would be easy to kill with biopesticide like any other available chemical brand of pesticides. Neonatal and first instar larvae were most susceptible to both Bt formulations, with susceptibility decreasing in 2nd and 3rd instars (Table 1). It might be expected that sensitivity to Bt toxin would decrease with larval age. The apparent increase in susceptibility of last instar larvae of *H. armigera* may be the result of increased feeding capacity at this stage, where a proportionally larger quantity of the treated diet

Table 2: ANOVA for significance among formulations on different growth stages of *H. armigera*

Sov	SS	D.F.	MS	F Ratio	Prob.
Formulations	36.125	1	36.125	2.131	0.2405
1st to 4th instars	7337.165	3	2445.722	144.248	9.678E-04
Error	50.865	3	16.955		
Total	7424.155	7			

Table 3: Lab potency of stored Bt biopesticide

Bt Formulations	Stored Time (months)	% mortality
A99	1	100
B99	3	98
C99	6	92
D98	12	92
E97	24	90
F96	36	94
Control	0	
Base only	0	

Table 4: Lab efficacy of stored Bt biopesticide against 2nd instar *H. armigera*

Bt formulation	Duration (months)	LC_{50} (µg/g of diet)
A99	0	83.0 ± 2.6
D98	12	85.3 ± 1.4
E97	24	86.4 ± 2.8

Table 5: *Helicoverpa armigera* larval population density in the plots before treatments.

Plot No.	Insect control agents	Number of larvae
1	Water	24
2	CAMB1-97	45
3	CAMB2-97	51
4	CAMB4-97	48
5	Formulation base only	30
6	CAMB1-98	57
7	CAMB2-98	36
8	CAMB4-98	69

was consumed by larvae. Some effects of sub-lethal doses of Bt on the 4th instar larvae were observed. The larvae did not die but the first movement slowed down with eventual paralysis conditions after taking the toxin. It is assumed that the 4th instar larvae may not always ingest sufficient quantities of Bt to be lethal. A reduction in weight gain among larvae treated with Bt was observed in the present experiments. The indications are that the toxin damaged the alimentary canal of the larvae which produced a general malaise and cessation of feeding leading to death (Karim *et al.,* 1999c). The pH and activity of digestive enzymes required to proteolytically activate the δ-endotoxin (Falcon, 1971) in the insect gut may also differ among different instars.

Bt inhibits larval feeding but at sub-lethal doses larvae may recover from initial exposure (Ali and Watson, 1982; Fast and Regniere, 1984) and continue feeding unless further treatment is applied. Bt is most effective against newly hatched larvae of *H. armigera*, a high percentage of which is present in tomato crop, persistence and stability of the material in spray is important for the effective control. Because of short half-life in field conditions, persistence may not contribute to multiple exposure of the recovering larvae or newly hatching larvae to active Bt. Therefore one week interval during application appear to be necessary for effective control of *H. armigera* on tomato if damaging populations persist. Bt treatment of longer durations also increased the cumulative larval mortality, indicating that the effects may only be low initially but the long term effects on a pest population could be more significant. This could be even more important in the field, where numerous mortality factors act on the population, and in larvae already weakened by Bt, the effect of such factors might be enhanced (Karim *et al.,* 1998).

In the present study, the results of application of Bt in the tomato crop indicated that the CAMB Bt formulation based on indigenous strain (Karim *et al.,* 1999a, 1999b) successfully controlled *H. armigera* in small scale tomato field after a storage period of 1-2 years (Table 6, 7). Therefore, this microbial control preparation may be considered as an alternative to the chemical pesticides used to control *H. armigera* in daily food crop like tomato. Exposure of Bt formulation also reduced the ability of *H. armigera* larvae to tunnel in tomato fruit. The differences in tunneling damage were significant, and would probably have been greater with higher concentrations of Bt or a longer period of exposure to Bt diet.

Preliminary field applications of Bt to first (asynchronous) generations of *H. armigera* resulted in relatively high numbers of

Table 6: Efficacy of Bt formulations after storage in contained conditions against the first population build up

Plot No.	Insect Control agents	Pre-sampling	7DAA (1) (% Mortality)	7DAA (2) (% Mortality)
1	Water	24	0	0
2	CAMB1-97	45	94*	100
3	CAMB2-97	51	99	100
4	CAMB4-97	48	100	100
5	Formulation base only	30	0	0
6	CAMB1-98	57	98	100
7	CAMB2-98	36	89*	97
8	CAMB4-98	69	98	99

LSD at 5% = 3.348 LSD at 1% = 4.469

Table 7: Efficacy of Bt formulations after storage in contained conditions against the second population build up

Plot No.	Insect Control agents	Pre-sampling	7DAA (1) (% Mortality)	7DAA (2) (% Mortality)
1	Water	21	0	0
2	CAMB1-97	12	22.0*	74.0*
3	CAMB2-97	70	99.5	99.5
4	CAMB4-97	14	89	100
5	Formulation base only	10	0	0
6	CAMB1-98	23	80	96
7	CAMB2-98	19	100	100
8	CAMB4-98	11	100	100

LSD at 5% =21.20 LSD at 1% = 27.74

surviving larvae feeding inside the tomato fruit, especially those of later instars. Nevertheless, the treatment halted further infestation and damage to the crop. However, the subsequent generation of *H. armigera* were synchronous and the microbial formulations were very effective when properly applied at the right time. It was most effective when most larvae were in their first or second instar growth stages. Therefore, precise pest scouting are of great importance.

Thus, CAMB Bt seems to have a significant and deleterious effect on *H. armigera* larvae. Field exposure of larvae to Bt showed decrease in feeding activity and by inference their tunneling capability, coupled with increased larval mortality, that would have a significant effect on reducing damage to field crops. It could also reduce survival of *H. armigera* population in the field from one season to the next.

Thus, all tested CAMB Bt formulations proved to be the most toxic and exhibited detrimental effects on the different stages of *H. armigera*. Therefore, CAMB Bt formulation can be used in integrated pest management of *H. armigera* without disrupting the agro-ecosystem and the quality of the environment. Further investigations on large scale field trials of CAMB Bt formulation on Lady's finger/Okra (*Abelmoschus esculentus* L. Moench) with *H. armigera* and *E. vitella* are underway.

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