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Effect of Bt toxin on development and cocoon characters of the silkworm, *Bombyx mori*

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Abstract
Effect of Bt toxin on the development and economic parameters of silk cocoons have been evaluated. Silkworm larvae at second, third, fourth and fifth instars inoculated with Bt spore commercial formulations having 5x107spore /mg. The amount of toxin ingested was about 0.025 µg, 0.050 µg, 0.25 – 0.375 µg, 1.75- 2.00µg and 2.475 – 2.5µg by second, third, fourth and fifth instars respectively. Late larvae ingested large quantity of Bt toxin resulting into acute disease and in the infected forth instar showed involuntary contraction of muscles, paralyzed and distressed. All the stages, second, third, fourth and fifth instars inoculated with Bt toxin exhibited weight loss due to infection during development. The highest mortality (90%) recorded at third instar larvae and lowest in second instar. Two fifth instar inoculated larvae were able to spin the cocoon but enabled to transform into pupa and died. During fifth instar LD50 was 2.38µg, which was approximately 68 times higher than required for second instar. Changes in the total body protein noticed on the second day of inoculation. Inoculation using different doses of Bt toxins, increased protein level drastically to 1.7 times in second, 2.2 times in third, 2.4 times in fourth and 1.7 times (128.3mg) in fifth instar treated as compared to 76.8mg in control.
Keywords: *Bacillus thuringiensis*, Bt toxin, silkworm, *Bombyx mori*

INTRODUCTION

Sericulture is the technique of silk production, an agro- industry playing an ambient role in the rural economy of India. Silk fiber is a protein produced from the silk glands of the silk worms. Historically sericulture was introduced for the first time in China. Among the developing countries like India, enjoys second favorable position and the current silk production of 18,000 MT tones owing to the low cost of labour. But sericulture industry suffers from a number of diseases, which causes severe damage to the industry.

Ishiwaki first discovered *Bacillus thuringiensis* in 1901, although its commercial importance was ignored until 1951. *B. thuringiensis* (Bt) is a rod shaped gram-positive bacterium isolated from several thousands of soil samples from 80 different countries. It is commonly present in aerial part of the plants such as leaves and on even washed fruits and vegetables we consume. Bt has been found in all types of terrain, including beaches, desert and tundra habitats (Travers et al., 1987). Bt is among the most thoroughly studied bacterial species of agricultural importance, and has been used on crops such as cotton, maize, soybeans, potatoes, tomatoes, various crops trees and stored grains. Over 100 species of bacterium that have been identified to cause disease in insects, only a few have been developed commercially. There are about 100 bio-pesticides exclusively based on *B. thuringiensis* have been produced and over 90 percent of the commercial bio-pesticide used even in organic forming. Bt based insecticide are applied to crop using conventional spraying technology. *B. thuringiensis* is a live microorganism that kills certain insect species and is used in forest, agriculture and urban areas. Bt itself is only mildly pathogenic but it produces intra cellular toxins that act as a gut poison, paralyses gut and death occurs due to septicemia. Bt is effective against lepidoptera, diptera (mosquitoes, house flies), coleoptera, nematodes, parasitic live flukes (Trematoda) and mites (Aeari) (Feitelson, 1993, Zukowski, 1995). Bt acts primarily at the larval stages have been used more intensively against lepidopteran pests. Bt variety *B. thuringiensis* subspecies *israelensis* that cause disease in mosquito and blackfly larvae (Margalit and Dean, 1985).

B. thuringiensis strain are usually classified on the basis of serological observation on the of delta endotoxins, its biological activity and size of the toxin protein (Hofte and Whiteley 1989) such as, CryI-Lepidopteran, CryII- Lepidopteran and dipteran, Cry III-Coleopteran, Cry IV-Dipteran, Cry V- Nematode, CryVI-Nematode Dipteran, Cry I-Dipteran.

The mechanism of action of Bt cry proteins involves solubilization of the crystal on insect mid gut proteolytic processing of the protoxin by mid gut protease, binding of activated crystal toxin to mid gut receptors and insertion of toxin into the apical membrane to create ion channels or pores. Crystals are comprised of protoxins to become active, susceptible insect must eat them. For most lepidopterans, protoxins are solubilized under the alkaline condition of insect mid gut (Gill, 1992, Krieg et al., 1983). The protease may be necessary for solubilization of toxin many protoxins must be processed by insect mid gut proteases to become activated toxin. These proteolytically processed protoxin to yield 60 KDa toxin core fragment. The toxin function is localized in the N- terminal half of the 130 KDa. Protein C-terminal half of these protoxin is highly conserved and is most likely involved in crystal formation.

Cry the activated toxin binds readily to specific receptor on the apical brush border of mid gut microvillae of susceptible insect causing strong damages to the epithelial mid gut cells (Aronson *et al.*, 1991). This is followed in a few hours by a general paralysis and death. In most cases, however larvae stop feeding within a few minutes of eating the toxin due to paralysis of gut. Some larvae may die immediately, but majority die from a septicemia two to three days later. Septicemia is caused by the escape of the *B. thuriengiensis kurstaki* spore through the damaged gut wall in the body cavity, where the spore germinates and the vegetative cell multiply (Aroson *et al.*, 1991).

In the present study the effect of Bt toxin on larval development, larval body protein and economic characters of cocoon of silkworm, *Bombyx mori* have been investigated.

MATERIALS AND METHODS

Sources of Bt Toxin :

B. thuringiensis produces a wide range of insecticidal toxins and their strains are usually classified on the basis of serological test and so for over 30 different serotypes have been identified. In this case commercial formulation of Bt server *kurstaki* subspecies serotype H3a, 3b, 3c, 5% WP having the concentration of 5×107 spore per mg. used for infectivity during second, third, fourth and fifth instar larvae of silkworm, *B. mori.*

Inoculation of Bt toxin

B. thuringiensis formulation a suspension having 5×107 spore / ml was prepared. The mass rearing of silkworm CSR2 x CSR4 was undertaken and about 200 newly molted healthy second, third, fourth and fifth instar larvae were selected from the stock culture. They were placed individually in plastic cups and divided in to two groups. All the larvae starved for about 10-12 hrs before the inoculation with *B. thuringiensis* toxin. About 25 larvae each from second, third, fourth and fifth instar were fed with a piece of mulberry leaf smeared with 2.5 µl of distilled water after air drying, and coded as IIA, IIIA, IVA and VA respectively. These groups and were used as control. Similarly 25 larvae each from second, third, fourth and fifth instars fed individually with a piece of mulberry leaf coated with 2.5 µl suspension of 5×107 spore /ml, coded as IIB, IIIB, IVB and VB respectively and used as treated groups.

The larvae that consumed whole piece of mulberry leaf were separated and further reared and maintained on fresh mulberry leaves upto cocoon formation. The mortality during development recorded in 24 h, 48h and 72h. The rearing conditions such as temperature and relative humidity were maintained according to the requirement of the different developing stage. A photo period of 14:10 light dark was maintained. Regular feeding, dusting of disinfectants, cleaning of beds and other rearing Schedule was followed as suggested by Krishnaswami (1978) and Dandin *et al.* (2003).

The quantity of leaves provided and consumed by larvae, incidence of diseases, mortality due to disease occurrence, period required for development and economic characters of cocoon were recorded.

Effect on Haemolymph Protein

Control and treated larvae were collected in eppendorff tube coated with phenylthiourea (to avoid coagulation of protein) and stored at – 20 oC deep freezer until use The control and treated larvae weighted and triturated in sample buffer, filtered and stored in the deep freezer. The total protein was estimated by Bradford (1976) method.

A stock solution of bovine serum albumin (BSA) (1mg/ml) was prepared and stored at 20 oC. Working solution was prepared by diluting the stock 10 times (0.1 mg/ml) Aliquots having 3800 distilled water were prepared and used as blank. Two aliquots each having 20, 40, 60, 80, 100 µg of

BSA in 80, 60, 40, 20 distilled water respectively were taken in eppendorff tube and total volume made upto 3800 µl in each tube using the 20µl protein sample of were taken in two aliquots each and total volume was made to 3800 µl using distilled water.

To the aliquots of blank, standard BSA and the samples having unknown amount of protein, about 200 µl Bradford's reagent was added to make up the total volume 4 ml. The samples were vortexed and optical density (O.D.) was measured at 595 nm using spectrophotometers (Elico- 175).

RESULTS

Ingestion of Bt toxin The second, third, fourth and fifth instars fed with 2.5 µg of Bt toxin/larvae, showed varied ingestion rate. The maximum number of larvae of second, third, fourth and fifth instar ingested about 0.025 µg, 0.050 µg, 0.25 – 0.375 µg, 1.75-2.00µg and 2.475 – 2.5µg respectively.

Symptomatology

The Bt toxin inoculated to second and third instar larvae, which ingested small quantity of Bt toxin caused chronic disease. Mulberry leaf intake is decreased, faeces became irregular in shape and muscle paralysis developed and larvae became motionless among the residual leaves as compared to control larvae. Forth and fifth instars larvae ingested large quantity of Bt toxin caused acute disease and in the infected forth instar involuntary contraction of muscles and paralyzed and distressed.

Larval development

The second, third, fourth and fifth instar larvae inoculated with Bt toxin exhibited weight loss due to infection during the development. Weight gained by the control larvae from first instar stage to prepupal stage. The weight of mature fifth instar larvae reached to 4.85 g /10 larvae in control group where it was 3.08 g/10 larvae when inoculated at fifth instar stage showed less weight as compared to control larvae (Table 1).

Mortality

Larvae of second, third, fourth and fifth stages when fed with Bt. Strain,the mortality recorded at different duration. The results indicate that, the larvae ingested high concentration of Bt died early and the larvae consumed less died at the later age. In second instar with lower consumption the mortality initiated from 48 hrs and by 96 hrs all died, whereas, with higher concentration, the death occurred within 24 hrs. Similarly, third and fourth instars died by 72 and 96 hrs respectively. During fifth instar with 2.25 to 2.5 µg equivalent of Bt. caused death of the larvae which was initiated at 24 hrs, evidenced by the dark brown region on the body surface of the larvae as compared to control and most of the larvae were dead by 9th day (Table 2). The highest mortality was recorded in third instar larvae and about 95% as compared to fourth, fifth and with lowest in second instar. Two fifth instar larvae were able to spin the cocoon but enabled to transform into pupa and died.

The comparison of different treated groups and control were made using ANOVA. In most of the treated groups $P < 0.001$ except in the group 23, 25, and 26 treatments where 5th and 3rd, 3rd and 4th and 4th and 2nd respectively (Table 3).

The lethal dose LD50 was evaluated for different stages, The LD50 for second instar was 0.035µg which increased to 0.3µg for third instar approximately 10 times to second stage. There after it increased by 6 times 1.75µg for fourth than that of previous stage (third instar). During fifth instar LD50 2.38µg, which was approximately 68 times higher than required for second instar (Table 4).

Haenolymph Protein

Proteins are important metabolites, which silkworm larvae obtained from mulberry diet. Economy of sericulture industry depends on growth of larvae and their ability to synthesize silk proteins required for good quality cocoons. This ability of procuring and synthesizing proteins is disrupted due to infection caused by harmful microorganisms during development. In the infected silkworm titre of pathogen level gradually increased and in consequence reflected on the body tissue, deprived of important metabolites essential for larval development, however the total body protein increased due to addition and sudden change in the physio- pathological conditions.

Changes in the total body protein noticed on the second day of inoculation. When larvae at different stages were inoculated using different doses of Bt toxins, the effect was quite drastic and instead of reducing the protein level increased drastically to 1.7 times in second, 2.2 times in third, 2.4 times in fourth and 1.7 times (128.3mg) in fifth instar treated as compared to 76.8mg in control (Table 5).

DISCUSSION

In Vidarbha generally cotton is main traditional crop, which attract many insect pests. To control various bioinsecticide formulations are used, which may be harmful to the useful insects such as silkworm and honey bees. Wild spread use of bioinsecticides require through investigation, and screening before being used in the field. *Bacillus thuringiensis u*sed in the commercial formulations against the cotton bollworm (Sreenivas *et al.*, 2002) which might contaminate the nearby crop of mulberry.

In the present study it was observed that the efficiency of concentration was effective resulted almost 90% mortality during second and third instar, where as late age larvae required higher dose of Bt Toxin to give similar results. Sreenivas *et al.* (2002) suggested the judicious use of these insecticides and reported 100% larval mortality when fed with drift sprayed leaves even after 30 days (Sreenivas *et al.*, 2002). Our results are also in agreement with the previous findings in which 100% mortality was reported by Ihran *et al.* (1993) and Saha (1994).

The present result further suggested that use even 2-2.5 µg killed larvae instantly and showed deleterious effect on gonads, such as testes and ovary which are the vital part of the system required for continuation of next filial generation. The endotoxin of Bt acted on the follicular epithelial cells lead to rapid destruction and disintegration of cell wall and cells. Saha *et al.* (1994) speculated that due to rapid loss epithelial cell caused loss of ATP from the cells stimulation increased respiration and glucose uptake, which ultimately lead to paralyses.

Endotoxin of Bt also acted quickly on the development and no moulting to the next stage was noticed and hence the mortality was evidenced as suggested by earlier workers (Shreenivas *et al.,* 2002, Krieg et al., 1983).

Therefore, it can be concluded that the commercial formulation of Bt server kurstaki subspecies serotype H3a, 3b, 3c, 5% WP used on insect pest of cotton in Vidarbha region particularly, may be harmful to the silkworm, *B. mori* and hence use of any biopesticide in the field should be avoided or minimized to avoid cross contamination and loss of silk crop in this region.

Table 1 : Effect of Bt toxin on larval weight during development of silkworm *Bombyx mori*.

Weight (g) / 10 larvae				
Instar	**Day**	**Control**	**Before treatment**	**After Death**
II	1	0.005 ± 0.002	0.004 ± 0.002	0.003 ± 0.002
III	1	0.019 ± 0.007	0.018 ± 0.005	0.011 ± 0.003
IV	1	0.111 ± 0.009	0.109 ± 0.019	0.096 ± 0.005
V	1	0.485 ± 0.011	0.484 ± 0.022	0.308 ± 0.011

Table 2 : Mortality obtained due to Bt toxin during development of silkworm, *B. mori*.

Sr. No.	Larval stages	Control / treated larvae	Dose (in µg)	No. of larvae used	Larval death within										
					2 hr	4 hr	24 hr	48 hr	72 hr	96 hr	7th day	8th day	9th day	Total larvae dead	% mortality
1	II	control	-	10	-	-	-	-	-	-	-	-	-	-	0.0
		Treated	0.1-0.4	10	-	-	-	4	3	3	-	-	-	10	90.0
			0.4-0.8	10	-	2	6	-	-	-	-	-	-	8	
2	III	Control	-	10	-	-	-	-	-	-	-	-	-	-	0.0
		Treated	0.1-0.4	10	-	-	7	2	1	-	-	-	-	10	95.0
			0.4-0.6	10		2	6	1	-	-	-	-	-	9	
3	IV	Control	-	25	-	-	-	-	-	-	-	-	-	-	0.0
		Treated	1.0-2.0	25	-	1	8	1	10	3	-	-	-	23	92
4	V	Control	-	25	-	-	-	-		-	-	-	-	-	-
		Treated	2-2.5	23	-	-	3	1	2	-	5	3	8	23	92

Table 3 : The comparison of different treated groups were analyzed using ANOVA is presented in table

Sr.No	Parameter	A	B	C
		Value	T 2nd	T 3rd
		Y	Y	Y
1	Table Analyzed			
2	Data 1			
3	One–Way analysis of variance			
4	P value	P<0.0001		
5	P value summary	***		
6	Are means signif. Different ?(P<0.05)	Yes		
7	No. of groups	5		
8	F	99.4		
9	R squared	0.950		
10				
11	ANOVA table	SS	Df	MS
12	Treatment (between columns)	27800	4	6940
13	Residual (within column)	1470	21	69.8
14	Total	29200	25	
15				
16	Newman-Keuls Multiple Comparison Test	Mean diff.	Q	P value
17	Control vs T 2nd	-95.0	24.9	P < 0.001
18	Control vs T 4th	-76.7	22.5	P < 0.001
19	Control vs T 3rd	-65.0	17.0	P < 0.001
20	Control vs T 5th	-53.3	15.6	P < 0.001
21	T 5th vs T 2nd	-41.7	10.9	P < 0.001
22	T 5th vs T 4th	-23.3	6.84	P < 0.001
23	T 5th vs T 3rd	-11.7	3.06	P < 0.05
24	T 3rd vs T 2nd	-30.0	7.18	P < 0.001
25	T 3rd vs T 4th	-11.7	3.06	P < 0.05
26	T 4th vs T 2nd	-18.3	4.81	P < 0.01

Table 4: Lethal Dose (LD 50) of Bt toxin to kill the larvae of different stages of silkworm, *Bombyx mori*.

Stage (Instar)	LD 50 (μg)
II	0.035
III	0.300
IV	1.750
V	2.375

Table 5 : Effect of Bt toxin on larval body protein during development of silkworm, *Bombyx mori*.

Stage	Age (Day)	Total body protein (mg / larva)	
		Control	Treated
II	2	12.6	21.2 *
III	2	16.6	36.5 *
IV	2	31.1	73.3 ***
V	2	76.8	128.3***

Significance: * = p < 0.05, ** = p<0.01, *** = p<0.001

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