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Research Article

Efficacy of Cotton Bollworm *Helicoverpa armigera* and Tobacco budworm *Heliothis virescens* against δ-endotoxin cry1ac and cry2ab expressed by genetically modified cotton plants

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Abstract

In present studies genetically modified cotton plant which express δ-endotoxin Cry1Ac and Cry2Ab were cultivated in 2012 and 2013. The efficacy was studied against cotton bollworm *Helicoverpa armigera* and tobacco budworm *Heliothis virescens.* Results of study suggest that Cry2Ab traits are effective against these species than Cry1Ac. The survival of *Helicoverpa armigera* was >90% on flower anthers and square anthers compared to 67% on flower bracts, flower petals, and whole squares from Cry1Ac expressing cotton plants at 72 hours. The expression of Cry2Ab was more effective against bollworms than Cry1Ac. On flower anthers and square anthers, expressed by Cry2Ab cotton plant survival of these worm found was 63% at 72 hours.

INTRODUCTION

Agricultural crops are attacked by several insects and pests. The world food production is adversely littered with insects and pests throughout crop growth, harvest and storage. The bollworm, *Helicoverpa armigera* (Hubner), occurs throughout India. It is a key pest of cotton plants and other crops in India and elsewhere, inflicting huge crop loss each year (Fakrudin *et al.*, 2004). Manjunath *et al.*, (1985) reported that cotton bollworm Helicoverpa armigera feeds on 181 cultivated and uncultivated plant species. Helicoverpa is known to cause economic losses to cotton, chickpea, tomato, pigeonpea and sunflower. The cotton bollworm *Helicoverpa armigera*, tobacco caterpillar *Spodoptera litura*, pink bollworm *Pectinophora gossypiella*, Jassids *Empoasca devastans* and whitefly *Bemisia tabaci* and are some of the major pests of cotton plants and having the potential to reduce the yields by 20-80%.

Cotton plants turn out various secondary plant chemicals that have an effect on insect development and survival (Hedin *et al.*, 1983). Maybe the foremost widely known and studied of those chemicals is that the sesquiterpenoid, gossypol (Stipanovic, 1983). Along with the gossypol, there are many other plant chemicals are also present in cotton plant. These include phenolics, catechin, anthocyanin flavonoids and tannins (White *et al.*, 1982). Organophosphorous and carbamate insecticides ultimately replaced chlorinated hydrocarbons due to this resistance. However, resistance to these compounds was reported within a few years as a result of widespread indiscriminate use (Wolfenbarger *et al.*, 1973).

Early season infestations rarely lead to economic loss in cotton production indicating that the current threshold is too low (Nimbalkar *et al.,* 2013). Recent advances in crop improvement have allowed the introduction of recombinant DNA from *Bacillus thuringiensis* Berliner var. kurstaki, that codes for the assembly of Bt (*Bacillus thuringiensis*) δ-endotoxin proteins into cotton plants and alternative crop plants (Perlak *et al.,* 1990). A *Bacillus thuringiensis* bacterium produces crystal proteins that are cytotoxic to many orders of blighter insects of economical and health importance (Aronson, A. I., and Y. Shai, 2001). The first Cry1Ac *Bacillus thuringiensis* Berliner (Bt) cotton variety was commercialized in 1996 (Bollgard®, Monsanto Ag. Co., St. Louis, MO, USA). In India, cultivation of Bt cotton is property as evident from the rise in space year when year from its industrial unleash within the year 2002-03 and it's well accepted by the farmers. During 2002-2003, genetically modified cotton plants were cultivated over an area of 72682 acres that enlarged to 213098 acres during 2003-04 and 1300000 ha throughout 2004-05.

MATERIALS AND METHODS

Transgenic Cotton Plants

Study plot was planted village Vihamandawa in Aurangabad district, in 2012 and 2013. In this period, two additional sites were planted near Nanded and Parbhani districts of Marathwada region of Maharashtra State, where cotton plantation area is very large. The experiment utilized the randomized complete block design with four cotton genotypes as treatments and four replications of each treatment. Depending on site, test plots were arranged from 8 to16 rows and were 13.7 meters long. Three test *Bt* cotton hybrids (NCEH 6), RCH 134 (Cry1Ac) and RCH 134 BGII (Cry2Ab) and NH44 non *Bt* cotton hybrids, were used for the study.

Insects

The Cotton Bollworm *Helicoverpa armigera* and Tobacco Budworm *Heliothis virescens* were obtained from colonies established in study plots.

Bioassays of Insects

Larvae were reared on artificial diet in individual vial. F_1 generation of *Helicoverpa armigera* and *Heliothis virescens* were used in Bioassay. Bioassays were performed to check the responses of *Helicoverpa armigera* and *Heliothis virescens* to terminal leaves, squares and bolls, of genetically modified cotton plants. During the process laboratory temperature was maintained at 25 ± 2°C and 75 ± 2 % relative humidity. Mortality of *Helicoverpa armigera* and *Heliothis virescens* larvae were recorded at 24 hrs, 48 hrs, 72 hrs and 96 hrs.

Estimation of Cry1Ac and Cry2Ab

δ-endotoxin Cry1Ac and Cry2Ab protein levels in the transgenic plants were analyzed by *Enzyme Linked Immunosorbent Assay* (ELISA).

RESULTS AND DISCUSSION

Sustainable expression of Cry1Ac and Cry2Ab in genetically modified cotton plants becomes very crucial for its effectiveness especially for the control of lepidopteran pests such as cotton bollworm and tobacco budworm were need to study, in our study we found that maximum mortality of these larvae was observed on leaves from 90 DAS plants. After 90 days, the mortality rate was declined; decreased mortality of these armyworms has been reported earlier (Shinde *et al.,* 2009; Nimbalkar *et al.,* 2011). Both the protein (Cry1Ac and Cry2Ab) found effective against bollworms than the other hybrid traits. However, in recent days, Cry2Ab had higher efficacy compared to Cry1Ac, but according to Adamczyk & Gore (2004) it report that Cry1F protein provided greater efficacy compared to Cry1Ac against this pest. In addition, previous studies have shown little efficacy of Cry1Ac against fall armyworms (Adamczyk *et al.,* 1998).

Efficacy of bollworm was varied among floral structures on NH44 (conventional). No cotton stage by floral structure (F<2.08; df =8, 45; P>0.06) or year by floral structure (F<0.59; df =4, 70; P>0.67) interactions were significant at any rating interval for efficacy of bollworm on NH44 cotton plant; therefore, data were combined across cotton stages and years. Survival averaged 93 to 100%, 81 to 98%, and 71 to 97% at 24hrs, 48hrs, and 72hrs after infestation, respectively (Table 1). At 24hrs, efficacy of bollworm was different among floral structures (F=4.37; df =4, 75; P<0.01). Efficacy of *Helicoverpa armigera* and Tobacco Budworm *Heliothis virescens* was lowest on flower bracts. Efficacy of bollworm at 48hrs (F=7.20; df =4, 75; P<0.01) and 72hrs (F=15.8; df =4, 75; P<0.01) was higher on flower anthers and square anthers than on flower bracts and petals. Efficacy of *Helicoverpa armigera* and Tobacco Budworm *Heliothis virescens* on anthers (flower and square) also was higher than on squares at 72hrs.

Efficacy of *Helicoverpa armigera* and Tobacco Budworm *Heliothis virescens* on Rasi 134 (Bollgard®) cotton varied among floral structures. No cotton stage by floral structure (F<1.43; df =8, 45; P>0.21) or year by floral structure (F<2.25; df =4, 70; P>0.07) interactions were significant for efficacy of bollworm at any rating interval; therefore, data were combined across cotton stages and years. Efficacy of bollworm ranged from 85 to 97%, 57 to 96%, and 19 to 91% at 24hrs, 48hrs, and 72hrs, respectively (Table 1). At 24hrs, efficacy was lower on flower bracts than all other structures (F=3.94; df =4, 75; P=0.01). At 48hrs (F=18.9; df =4, 75; P<0.01) and 72hrs (F=71.3; df =4, 75; P<0.01), efficacy of bollworm was higher on flower anthers and square anthers than on other floral structures. There were no differences between efficacy of bollworm on Rasi 134 and Rasi 134 BGII for any structure at 24hrs (Table 1). At 48hrs, survival of *Helicoverpa armigera* was lower on Rasi 134 flower bracts and squares compared with the corresponding structures on Rasi 2000. Efficacy of bollworm was lower on all Rasi 134 BGII structures compared with the corresponding structures on Rasi 134 at 72hrs.

Enzyme Link Immunosorbent Assay (ELISA) tests of floral structures used in these bioassays indicate that *Bacillus thuringiensis* protein expression varies among plant parts (F=32.6; df =4, 10; P<0.01). Protein expression was highest in flower bracts and petals compared with other structures. In addition, protein expression was lowest on squares and square anthers. Cry1Ac expression averaged (± standard deviation) 0.59±0.03, 0.56±0.12, 0.34±0.03, 0.17±0.03, and 0.19±0.01 ppm on flower bracts, flower petals, flower anthers, square anthers, and squares, respectively. Cry1Ac levels did not correlate (24hrs: R=-0.21; F=0.63; df=1, 13; P=0.44; 48hrs: R=-0.30; F=1.33; df=1, 13; P=0.27; 72hrs: R=-0.29; F=1.18; df=1, 13; P=0.30) with variation in *Helicoverpa armigera* survival.

Efficacy of *Heliothis virescens* on Floral Components of cotton plants expressing Cry1Ac and Cry2Ab

Survival of *Heliothis virescens,* on flower anthers and square anthers was generally highest and lowest, respectively, on flower bracts on NH44, RASI 134, and RASI 134 BGII (Table 2). *Heliothis virescens* survival on against Cry2Ab appeared to follow a trend similar to that observed on Bollgard®. However, it was much lower on Cry2Ab expressing cotton pant than Cry1Ac expressing cotton plants. At 24hrs, there were no differences in efficacy of bollworm among the three cotton plant cultivars on any structure (Table 2). At 48hrs, efficacy of bollworm on squares was lower on Cry2Ab expressing cotton pant than on squares from the other cotton plant cultivars. Efficacy of bollworm at 72hrs was lower on all flower structures from Cry2Ab expressing cotton pants than on the corresponding structures on NH44 and RASI 134 cotton plant cultivars.

In study, *Helicoverpa armigera* larvae showed a greater preference than *Helicoverpa virescens* larvae for white flowers. Non-photosynthesizing (non-green) structures of cotton plant may be more common feeding sites for bollworm larvae. These structures, which are mostly reproductive, may be more nutritionally suitable for bollworm larvae than other plant parts. Another possible explanation for bollworm preferences for flowers could be that there are lower levels of secondary plant chemicals in non-photosynthesizing tissues. Dose-mortality regressions in this study indicate that cotton bollworm populations in Marathwada region may be less susceptible to pyrethroids than previously thought (Nimbalkar *et al.,* 2012). According to Hedin *et al.,* (1983) varying levels of secondary plant chemicals (tannins, gossypol, and chrysanthemin) among different plant parts. Gossypol concentrations ranged from 0.04% in bolls to 0.50% in squares. Tannins ranged from 6.02% in terminals to 17.1% in bolls, while chrysanthemin ranged from 0.05% in bolls to 0.18% in leaves. Stipanovic (1983) reported that cotton plant foliage produces numerous terpenoids and other compounds in addition to gossypol. Many of the compounds found in cotton plant have antibiotic activity and are toxic to several insect pests. Little information is available concerning levels of secondary plant chemicals in square anthers. Hanny (1980) reported variation in levels of selected chemicals in flower anthers among cotton plant cultivars. Also, yellow flower anthers contained more gossypol than cream-colored flower anthers. Studies comparing the concentrations of secondary chemicals in flower anthers to those in other plant parts have not been conducted. It is likely that bollworm mortality on flower structures is associated with more than one allelochemical within an individual structure and differences in chemical complexes among cotton plant parts may explain the variation in efficacy of bollworm on those plant parts.

Table 1 Comparisons of efficacy of Cotton Bollworm *Helicoverpa armigera* and Tobacco Budworm *Heliothis virescens* at 24, 48, and 72hrs after infestation with neonates on NH44 and Rasi 134 floral components.

		Mean (±SD) % Survival				
24h	**Floral Structure**	**NH44**	**Rasi 134**	***df***	***f***	***P>f***
	Bracts	93 ± 8A	85 ± 15B	30	-1.82	0.08
	Petals	96 ± 4ABC	94 ± 7A	30	-1.23	0.23
	Flower Anthers	98 ± 5AB	97 ± 7A	30	-0.4	0.69
	Square Anthers	100 ± 0A	97 ± 10A	30	-1.34	0.19
	Squares	95 ± 6BC	93 ± 8A	30	-1.01	0.32
	F	4.37	3.94			
	df	4,75	4.75			
	P>F	<0.01	0.01			
48h	**Floral Structure**	**NH44**	**Rasi 134**	***df***	***f***	***P>f***
	Bracts	81 ± 16C	57 ± 21D	30	-3.74	<0.01
	Petals	89 ± 12BC	82 ± 13B	30	-1.53	0.14
	Flower Anthers	98 ± 5A	96 ± 4A	30	0.9	0.38
	Square Anthers	98 ± 6A	94 ± 10A	30	-1.32	0.2
	Squares	91 ± 8AB	70 ± 21C	30	-3.71	<0.01
	F	7.2	18.9			
	df	4.75	4,75			
	P>F	<0.01	<0.01			
72h	**Floral Structure**	**NH44**	**Rasi 134**	***df***	***f***	***P>f***
	Bracts	71 ± 18C	19 ± 15D	30	-8.76	<0.01
	Petals	72 ± 12BC	58 ± 15B	30	-3.67	<0.01
	Flower Anthers	97 ± 5A	91 ± 6A	30	-2.59	0.01
	Square Anthers	96 ± 6A	88 ± 9A	30	-2.87	0.01
	Squares	83 ± 12B	97 ± 23C	30	-7.12	<0.01
	F	15.8	71.3			
	df	4,75	4,75			
	P>F	<0.01	<0.01			

Means within columns followed by a common letter are not significantly (α=0.05) different according to Fisher's protected least significant difference. Means within rows are compared using paired t-tests (α=0.05).

Table 2 Mean (± standard deviation) efficacy of bollworm on NH44, Rasi 134 (Bollgard®), and Rasi 134 BGII (Bollgard® II) floral structures at 24 hrs, 48 hrs, and 72 hrs after infestation

		Mean (±SD) % Survival					
24hrs	**Floral Structure**	**NH44**	**Rasi 134**	**Rasi 134 BGII**	***F***	***df***	***P>f***
	Bracts	83 ± 13Aa	80 ± 13Ba	89 ± 3Ba	0.66	2, 8	0.54
	Petals	98 ± 3Aa	100 ± 0Aa	99 ± 3Aa	0.62	2, 8	0.56
	Flower Anthers	98 ± 3Aa	100 ± 0Aa	99 ± 3Aa	0.68	2, 8	0.53
	Square Anthers	98 ± 3Aa	100 ± 0Aa	100 ± 0Aa	1.45	2, 8	0.29
	Squares	85 ± 6Aa	96 ± 4Aa	97 ± 4Aa	2.09	2, 8	0.19
	F	2.39	7.84	10.49			
	df	4,10	4.15	4, 15			
	P>F	0.12	<0.01	<0.01			
48hrs	**Floral Structure**	**NH44**	**Rasi 134**	**Rasi 134 BGII**	***F***	***df***	***P>f***
	Bracts	67 ± 7Ca	57 ± 23Cb	29 ± 19Cb	4.16	2, 8	0.06
	Petals	89 ± 12BC	90 ± 10ABa	81 ± 15Aa	1.28	2, 8	0.33
	Flower Anthers	98 ± 5A	98 ± 3Aa	88 ± 17Aa	0.43	2, 8	0.3
	Square Anthers	98 ± 6A	97 ± 3Aa	72 ± 19Ab	6.18	2, 8	0.02
	Squares	91 ± 8AB	77 ± 12Ba	38 ± 28Bb	5.2	2, 8	0.04
	F	11.1	7.39	6.89			
	df	4, 10	4, 15	4, 15			
	P>F	<0.01	<0.01	<0.01			
72hrs	**Floral Structure**	**NH44**	**Rasi 134**	**Rasi 134 BGII**	***F***	***df***	***P>f***
	Bracts	48 ± 9Ca	18 ± 6Db	6 ± 2Cc	42.7	2, 8	<0.01
	Petals	81 ± 9Aba	67 ± 13Ba	36 ± 21Bb	7.58	2, 8	0.01
	Flower Anthers	95 ± 5Aa	93 ± 2Aa	63 ± 9Ab	33.3	2, 8	<0.01
	Square Anthers	97 ± 5Aa	92 ± 3Aa	50 ± 10ABb	49.9	2, 8	<0.01
	Squares	75 ± 17Ba	49 ± 14Cb	8 ± 4Cc	25.9	2, 8	<0.01
	F	11.2	45.8	19.9			
	df	4, 10	4, 15	4, 15			
	P>F	<0.01	<0.01	<0.01			

Means within a column followed by the same uppercase letter and within a row followed by the same lowercase letter are not significantly (α=0.05) different.

Differences in *Bacillus thuringiensis* Cry1Ac protein expression among different plant parts may partially explain differences in efficacy of bollworm on those structures. However, similar differences in efficacy of bollworm among floral structures were observed on conventional cotton plant, which indicates that factors other than protein expression alone are involved. For example, interactions between plant secondary compounds and the Cry1Ac protein may have occurred. If there is an interaction between Cry1Ac and plant allelochemicals, then there would be an expected minimum critical level of protein that fluctuates based on allelochemical concentrations.

For instance, structures with low allelochemical concentrations would require a higher level of Cry1Ac expression to provide the same level of bollworm mortality as structures with high allelochemical concentrations. Therefore, the interactions of these factors would be dynamic, where a decrease in one factor may require an increase in the other factor to provide the same level of protection.

Although statistical differences were observed between conventional and Bollgard® cotton plant, efficacy of bollworm averaged ≥88% on Bollgard® flower anthers and square anthers. With this level of pest pressure, insecticide applications may be needed to prevent economic losses. Differences in efficacy of bollworm on conventional and Bollgard® cotton plant support the presence of Cry1Ac protein in those structures of Bollgard® cotton plant with high levels of efficacy of bollworm. However, expression in those structures may be low. Bollgard® II contains an additional gene that codes for the production of the Cry2Ab protein from *Bacillus thuringiensis* in addition to Cry1Ac. The addition of the Cry2Ab protein with Cry1Ac increased the insecticidal activity against *Helicoverpa armigera*. Sims (1997) reports that bollworm larvae appear to be less sensitive to Cry2Ab than Cry1Ac. The addition of the Cry2Ab protein into Bollgard® cotton plant, however, would most likely increase the total amount of protein present in the plant. Greenplate *et al*., (2000) measured levels of Cry proteins present in Bollgard® II. They found approximately a 10X higher level of Cry2Ab over Cry1Ac; however, there was only a 3-6X increase in bioactivity against *Helicoverpa virescens*. In the present study, increases in bioactivity against bollworms of 3.2X, 1.6X, 1.4X, 1.8X, and 4.6X for flower bracts, flower petals, flower anthers, square anthers, and squares, respectively, were observed. Still in India, Bollgard® cotton plant cultivars are valuable integrated pest management tools for cotton plant cultivation. Good control can be expected for the *Helicoverpa virescens* (Tobacco Bud Worm) and pink bollworm, *Pectinophora gossypiella* (Saunders). This new technology has not always provided sufficient levels of bollworm control, however. Data reported here support field observations made by agricultural consultants and researchers throughout south India, concerning high numbers of bollworm larvae feeding on white flowers. It was originally assumed that white flowers express lower levels of Cry1Ac protein than other plant parts. However, other factors may be involved based on the ELISA (Enzyme-linked Immunosorbent Assay) data and efficacy of bollworm trends on conventional cotton plant floral structures.

Similar trends in efficacy of bollworm were observed on conventional and Bollgard® floral structures. Significantly fewer larvae survived on flower bracts of conventional cotton plant compared with survival on other conventional cotton plant floral structures. This finding suggests that biochemical factors associated with bracts have adverse effects on bollworm development. The addition of a second protein into Bollgard® cotton plant to create Bollgard® II appeared to significantly increase protection against bollworms. Despite these improvements, however, efficacy of bollworm averaged over 50% on flower anthers and square anthers of Bollgard® II at 72hrs. These survival rates suggest that economic injury may occur on Bollgard® II during bollworm outbreaks; however, these experiments were terminated after 72hrs. Our data suggest that the possibility for injury exists, but this has not been observed for Bollgard® II cotton plant grown under field conditions. Field studies report that Bollgard® II cottons will consistently provide satisfactory bollworm control (Jackson *et al*., 2000, Stewart and Knighten 2000, Ridge *et al*., 2000). However, these were small plot studies conducted in relatively isolated locations and no definitive predictions can be made as to the level of bollworm protection that can expected from Bollgard® II when it is planted over large acreages. In conclusion, these data provide a baseline of information describing the levels of efficacy of bollworm that can be expected on white flowers of Bollgard® and Bollgard® II cotton plant. This information indicates that current scouting protocols for conventional cotton plant may not be appropriate for Bollgard® cotton plant. Because high levels of efficacy of bollworm can be expected on white flowers of Bollgard® cotton plant, those structures need to be closely examined for small larvae. Also, these data provide valuable information for improving management decisions for bollworms on Bollgard® cotton plant.

Further research is needed to determine if larvae feeding on white flowers are capable of moving to other structures, causing additional injury. Also, future research in this area should focus on quantifying secondary plant chemicals and assessing nutritional quality among selected components of white flowers and squares to

determine their influence on Cry1Ac efficacy. Finally, *Helicoverpa armigera* management in genetically modified cottons (Bollgard® and Bollgard® II) is a complex situation that involves multiple factors. Plant biochemistry and nutrition appear to be important for bollworm mortality, in addition to *Bacillus thuringiensis* protein expression in genetically modified cottons. Levels of secondary plant chemicals and *Bacillus thuringiensis* protein expression need to be determined for different genetically modified cultivars and among different plant parts so that efficacy of bollworm can be predicted during periods of high population densities.

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