Baseline Susceptibility of American bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera : Noctuidae) to the Cry2Ab2 Protein from *Bacillus thuringiensis*

Report
2003-2004

MAHARASHTRA HYBRID SEEDS COMPANY, LTD
Resham Bhavan, 4th Floor, 78 Veer Nariman Road, Mumbai 400 020, India

March 2004

CONTENTS

Baseline Susceptibility of American bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera : Noctuidae) to the Cry2Ab2 Protein from *Bacillus thuringiensis*

Abstract

The susceptibility of Indian populations of *H. armigera* (Hubner) to the insecticidal protein Cry2Ab2, from *Bacillus thuringiensis* Berliner was determined through insect feeding bioassays (Diet incorporation method). Populations of *H. armigera* were collected from cotton fields during the cropping season of 2003 from major cotton farming areas of Central India (Jalna, Khandwa and Vadodora) and South India (Haveri, Coimbattore and Rangareddy). All the populations tested were susceptible to Cry2Ab2. The lethal concentrations (related to mortality), LC_{50} ranged from 1.399 to 2.152 in Central India and 2.201 to 2.501 µg Cry2Ab2 per ml of diet in South India. The moult inhibitory concentrations (related to developmental stage, not allowing larvae to go beyond 1st instar), MIC_{50} sensitivity ranges for Central and South Indian cotton area were 0.345 to 0.854 and 0.518 to 0.933 µg of Cry2Ab2 per ml of assay diet, respectively. The Inhibitory concentration (not allowing the larvae to reach 3rd instar), IC_{50}, ranged form 0.068 to 0.322 in Central India and 0.077 to 0.181 µg of Cry2Ab2 per ml of assay diet in South India. The effective concentrations (weight related) EC_{50} sensitivity ranges for Central and South Indian cotton area were 0.018 to 0.055 and 0.032 to 0.038 µg of Cry2Ab2 per ml of assay diet, respectively. The variability in toxicity during the initial comparison (baseline) was to an extent of 2 and 3- fold at the LC_{50} and EC_{50} to Cry2Ab2 protein. The data included in this report have provided important information on the susceptibility of *H. armigera* field strains to Cry2Ab2 protein before the commercialization of Bollgard II® in India. This will allow development of diagnostic doses which would be more efficient in detection of *H. armigera* populations resistant to Cry2Ab2 toxins. These bioassay parameter values will represent the appropriate doses for future routine monitoring of resistance to Cry2Ab2 toxin through discriminating dose assays. However, it is important to ensure that appropriate *Bt* cotton cultivation strategies must be designed to ensure the survival of susceptible insects which would protect the technology from becoming obsolete due to evolution of insect resistance.

1. INTRODUCTION

Advances in genetic engineering including insertion and expression of two *Bt* toxin genes with a broader spectrum activity have resulted in gene pyramiding which allowed the development of Bollgard II® with increased potency against target pests and broaden the spectrum of total bollworm pests controlled. A potential advantage of having two *Bt* toxins expressed in cotton is that the risk of insect pests developing resistance to *Bt* cotton may be reduced, especially if resistance to one toxin does not confer resistance to the other. Although not yet commercially available in India, transgenic cotton lines expressing two *Bt* toxins are under development. Once Bollgard II® cotton which have been genetically modified to express two types of delta -endotoxins from *Bacillus thuringiensis* (both Cry1Ac and Cry2Ab2) are commercially available, could play an important role in Integrated Pest Management (IPM).

The extent of both inter- and intra-population natural variation in susceptibility to a given *Bt* – based product should be investigated before biologically important changes can be identified with any certainty. Ideally, this should be done before the product is used commercially rather than after resistance is already widespread. Knowledge of the natural variation in response to Cry2Ab2 among *H. armigera* populations before widespread commercial use of Bollgard II® is necessary to avoid unwarranted concerns about resistance to Cry2Ab2 in field surveys of *H. armigera* populations. The development and implementation of effective resistance monitoring programs capable of early detection of resistance will allow implementation of appropriate management decisions in a timely manner (Dennehey, 1987). The initial steps in implementing such programs include establishment of baseline susceptibility data among populations across the geographical range of the target species. With this information, potential population susceptibility changes in response to selection with *Bt* toxin can be identified. The objective of the current study was to establish a baseline susceptibility to Cry2Ab2 toxin from geographically distinct populations of *H. armigera* collected from cotton, emphasizing areas where Bollgard II® cotton would potentially be commercialized.

2.MATERIALS AND METHODS

2.1 SAMPLING REGIONS AND FIELD STRAINS

Laboratory strains of *H. armigera* were established from larvae collected in cotton fields during the cropping season of 2003-04 from major cotton growing regions of Central and South India. Field strains of cotton bollworm were collected during September-October 2003, in cotton fields from three districts of Central India viz., Jalna (Maharashtra), Khandwa (Madhya Pradesh) and Vadodora (Gujarat) and three districts of South India viz., Haveri (Karnataka), Coimbattore (Tamil Nadu) and Rangareddy (Andhra Pradesh).

Third to sixth instar *H. armigera* larvae were collected from cotton plants. At least 300 larvae were collected from each site and were reared to the pupal stage in the laboratory on artificial diet based on chickpea flour. Emerging adults were transferred to mating chambers with male:female ratio

of 1:1 and were fed with 10% honey solution. The inner surface of mating chamber was covered with a muslin cloth for oviposition. Eggs were collected every 24 hours during the oviposition period. The cloth strips with eggs were incubated individually in 500 ml clear glass jars and were allowed to hatch. The resulting neonate larvae were used in bioassays to determine the Cry2Ab2 susceptibility and the rest were used to carry-over the rearing process into next generation on artificial diet to maintain cultures.

2.2 Bt insecticidal protein

Susceptibility of *H. armigera* to Cry2Ab2 was determined with either F1 or F2 generation in the laboratory by Diet-incorporation method. Plant-derived tissue powder expressing 6.014 mg of Cry2Ab2 per gm was used as standard source of Cry2Ab2 in the bioassays.

2.3 Bioassay procedures

The concentration response assays were performed using diet incorporation method in a manner similar to that described by Sims *et al.* (1996). Semi-synthetic diet for *H. armigera* was prepared and poured into sterile glass beakers and kept warm in a hot water bath maintained at 55°C. The primary stock solution for Cry2Ab2 was prepared by thoroughly mixing 207.8 mg toxin in 5 ml of 0.2% agar solution by using 'Thermolyne' maxi mix. Seven serial dilution's were prepared sequentially in 0.2% agar solution in sterile oak ridge centrifuge tubes (50 ml) by diluting it to 1/3 of the previous concentration. The concentrations bioassayed were 50.00, 16.667, 5.556, 1.852, 0.617, 0.206 and 0.069 µg of Cry2Ab2 / ml of diet. Different concentrations of the toxin solutions were mixed thoroughly into warm semi-synthetic diet pre-cooled to 55°C, at a rate of 2.8 ml of the toxin solution per 11.2 ml diet and 0.75 ml of each concentration dispensed into 128-well insect assay trays. Newly hatched, active larvae were released at the rate of one larvae per well at a total of 16 larvae per concentration in 5 replicates of each geographical population on the diet incorporating different concentrations of the toxins. After larval transfer, the assay trays were covered with self-adhesive tabs and ventilated with a single insect pin hole. All the assays were performed in the laboratory using seven concentrations plus one untreated control and incubated at conditions of 27 ± 1°C and 70% relative humidity.

Observations were recorded on mortality, stadia and weight of surviving larvae in each concentration on the eighth day. Observed mortality was corrected with respect to mortality in control group. Various bioassay parameters like lethal concentrations[1] (LC_{50} and LC_{90}); moult inhibitory concentrations[2] (MIC_{50} and MIC_{90}); inhibitory concentrations[3] (IC_{50} and IC_{90}) and Effective concentrations[4] (EC_{50} and EC_{90}) with confidence limits were computed for each assay.

1.**Lethal concentration, LC_{50}** - Dose of Cry2Ab2 in µg needed to kill 50% of the test larval population in an assay period of 7 days. Similarly LC_{90} is a dose needed to kill 90% of the test population.

2.**Moult inhibitory concentration, MIC_{50}** - Dose of Cry2Ab2 in µg that will inhibit molting of neonates into 2nd instar, of 50% of test population in the assay period of 7 days. MIC_{90} is a dose of Cry2Ab2 that will inhibit molting of 90% of test larval population.

3.**Inhibitory concentrations, IC_{50} and IC_{90}** Doses of Cry2Ab2 in µg that will inhibit molting of 2nd instar larvae into 3rd instar in 50 and 90% of test population, respectively in the assay period of 7 days.

4.Effective concentration, EC_{50} and EC_{90} . Dose of Cry2Ab2 in µg that would stunt the larvae so as to weigh 50% and 90% of the weight of untreated control larvae, respectively.

Probit analysis of the data (Finney, 1971) for each geographical population was carried out using the POLO-PC statistical package (LeOra Software, 1987) to compute LC_{50}, LC_{90} (mortality related), MIC_{50}, MIC_{90} (Moulting related), IC_{50}, IC_{90} , EC_{50} and EC_{90} (related to Weight reduction). One-way analysis of variance (ANOVA) was carried out to compare estimates for different populations and means were separated by LSD / CD values whenever the ANOVA was significant.

3. RESULTS

3.1 Mortality response evaluation

The results of mortality response evaluation among *H. armigera* populations are illustrated in Table 1. The median lethal concentration (LC_{50}) values ranged from 1.399 to 2.501 µg of Cry2Ab2 / ml for the six populations screened by diet incorporation assay. The sensitivity ranges for Central Indian cotton area (Khandwa, Jalna and Vadodara) and South Indian cotton area (Haveri, Rangareddy and Coimbattore) were 1.399 to 2.152 µg of Cry2Ab2 / ml and 2.201 to 2.501 µg of Cry2Ab2 / ml, respectively. The LC_{90} values were between 19.777 to 47.809 µg of Cry2Ab2 / ml. Significant differences in susceptibility were not detected among the populations tested and overall, the range of sensitivities of these six strains was just under 2- and 2.5- fold for LC_{50} and LC_{90}, respectively.

3.2 Growth inhibition response evaluation

The larval growth inhibition data for different strains of *H. armigera* to the Cry2Ab2 protein is presented in Table 2 and 3. The disparity of MIC_{50} values, at the concentration causing 50% of inhibition of growth to the 2nd instar ranged from 0.345 (Khandwa) to 0.933 µg of Cry2Ab2 / ml (Haveri); and the variation of sensitivity to Cry2Ab2 protein was nearly 3-fold. The MIC_{90} values were between 2.715 to 6.779 µg of Cry2Ab2 / ml with a similar variability between lowest (Khandwa) and highest (Haveri) being 2.5-fold.

The Cry2Ab2 instar stunting responses, IC_{50}'s, at the concentration causing 50% of inhibition of growth to the 3rd instar ranged from 0.068 (Khandwa) to 0.322 µg of Cry2Ab2 / ml (Vadodara); the latter IC_{50} was nearly 5 times as high as high as the former, showing that variation of this bioassay parameter is more than those indicated by mortality. The IC_{90} values were in the range of 0.221 to 1.422 µg of Cry2Ab2 / ml indicates a high variability in response with the difference between lowest (Coimbatore) and highest (Vadodara) being 6.5-fold.

3.3 Weight stunting response evaluation

The Cry2Ab2 weight stunting concentration responses, EC is the concentration which would stunt the weight of treated population from reaching half the average weight of control population. The EC_{50} values ranged from 0.018 to 0.055 µg of Cry2Ab2 / ml for the six populations screened whereas

the EC_{90} values ranged from 0.122 to 0.412 µg of Cry2Ab2 / ml (Table 4) for *H. armigera* in this study. According to EC_{50} values, the Jalna population was three times more susceptible than the rest of the populations of *H. armigera* screened.

4. DISCUSSION

One of the important factors that can influence the efficacy of *Bt* transgenic crops for *H. armigera* management is the variability in susceptibility to the Cry toxins in different populations across the country. Although variation in susceptibility to Cry2Ab2 was observed, the magnitude of the differences was small (i.e. 2- and 3- fold at the LC_{50} and EC_{50}) and similar to other estimates of baseline variability among geographically distinct populations of other insect species. However, It cannot be assumed that these data represent the complete range of susceptibilities found among *H. armigera* populations in India, but they do show a relative complete and realistic range of normal responses. Likewise, geographical variation in susceptibility to Cry1Ac through baseline susceptibility studies was earlier reported for *H. armigera* in China (Wu et. al., 1999) and the related species, *H. virescens* (4-fold) and *H. zea* (16-fold) in USA (Stone and Sims, 1993) and found considerable inter-population variation in Cry1Ac susceptibility. However Sims et al. (1996) suggested that this inter-population variation in Cry1Ac susceptibility may reflect non-genetic variation or sampling error, because the populations tested represented a small sample, taken at one point of time, of considerably larger multivoltine populations.

The data included in this report have provided important information on the susceptibility of *H. armigera* field strains to Cry2Ab2 protein before the commercialization of Bollgard II® in India. Continued collection and analysis of these kinds of data are critical to the development and continual assessment of resistance management strategies.

5. CONCLUSION

Development of baseline susceptibility data is a prerequisite to the development of a monitoring program designed to detect changes in susceptibility that may result from repeated and prolonged exposure to Bt toxins. These data also may provide information that will allow development of diagnostic bioassays that would be more efficient in detection of populations resistant to *B. thuringiensis* toxins. Monitoring for potential resistance in *H. armigera* populations would be facilitated by the establishment of such diagnostic doses, and future efforts should focus on the determination of diagnostic doses for effectively detecting any significant shifts in susceptibility profiles of *H. armigera* field populations to Cry2Ab2 post- Bollgard II® commercialization.

6. REFERENCES

Dennehey, T. J. 1987. Decision-making for managing pest resistance to pesticides. pp. 118-126. *In* M. G. Ford, D. W. Holloman, B. P. S. Khanbay and R. M. Sawicki [eds.], Combating resistance to xenobiotics: biological and chemical approaches. Ellis Horwood, Chichester, England.

Finney, D. J. 1971. *Probit analysis*. (3rd Ed.), Cambridge University Press. 318 pp

Le Ora Software. 1987. POLO – PC. A user's guide to probit and logist analysis. Le Ora Software, Berkely, CA.

Sims, S. R., J. T. Greenplate, T. B. Stone, M. A. Caprio and F. L. Gould. 1996. Monitoring strategies for early detection of Lepidoptera resistance to *Bacillus thuringiensis* insecticidal proteins. In: Brown T. M. (Ed), *Molecular genetics and evolution of pesticide resistance*, American Chemical Society Symposium Series. No. 645, pp. 230 – 242.

Stone, T. B. and S. R. Sims. 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera : Noctuidae) to *Bacillus thuringiensis*. *J. Econ. Entomol.* 86: 989 – 994.

Wu, K., Y. Guo and N. Lv .1999. Geographic variation in susceptibility of *Helicoverpa armigera* (Lepidoptera : Noctuidae) to *Bacillus thuringiensis* insecticidal protein in China. *J. Econ. Entomol.* 92: 273 – 278.

Table1 : Concentration - responses of *H. armigera* larval mortality (LC) to Cry2Ab2 protein

Location	State	LC_{50} (µg/ml)	95% Fiducial limits		LC_{90} (µg/ml)	95% Fiducial limits		Slope ± SE
			Lower	Upper		Lower	Upper	
Central zone								
Khandwa	M.P	1.399[a]	1.016	1.846	21.554[a]	13.054	32.647	1.115 ± 0.089
Jalna	Maharashtra	1.627[a]	1.093	2.162	19.777[a]	10.978	35.403	1.199 ± 0.084
Vadodara	Gujarat	2.152[a]	1.381	3.108	21.309[a]	11.808	40.996	1.320 ± 0.103
South zone								
Haveri	Karnataka	2.201[a]	1.508	2.816	35.434[a]	19.377	51.105	1.114 ± 0.096
Rangareddy	AP	2.501[a]	1.655	3.057	47.809[a]	22.684	62.272	1.076 ± 0.089
Coimbattore	Tamil Nadu	2.413[a]	1.743	3.005	28.603[a]	17.917	43.085	1.210 ± 0.095
SEM		0.41			10.42			
CV %		44.28			80.10			
LSD (P= 0.05)		1.18			30.40			

@LC_{50} & LC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits

Table 2 : Moult inhibitory concentration (MIC) responses of *H. armigera* to Cry2Ab2 protein

Location	State	MIC_{50} (µg/ml)	95% Fiducial limits		MIC_{90} (µg/ml)	95% Fiducial limits		Slope ± SE
			Lower	Upper		Lower	Upper	
Central zone								
Khandwa	M.P	0.345[ab]	0.225	0.488	2.715[ab]	1.687	5.075	1.440 ± 0.122
Jalna	Maharashtra	0.802[a]	0.616	0.928	4.601[a]	3.314	6.294	1.675 ± 0.122
Vadodara	Gujarat	0.854[a]	0.566	1.121	4.404[a]	2.760	7.540	1.801 ± 0.145
South zone								
Haveri	Karnataka	0.933[a]	0.595	1.238	6.779[a]	3.938	10.438	1.555 ± 0.136
Rangareddy	AP	0.796[a]	0.441	0.982	5.330[a]	3.554	10.655	0.441 ± 0.982
Coimbattore	Tamil Nadu	0.518[a]	0.281	0.616	2.835[ab]	2.114	4.359	1.423 ± 0.116
SEM		0.16			1.25			
CV %		49.68			62.84			
LSD (P= 0.05)		0.46			3.65			

@MIC_{50} & MIC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits

Table 3 : Inhibitory concentration (IC) related to instar stunting responses of *H. armigera* to Cry2Ab2

Location	State	IC_{50} (µg/ml)	95% Fiducial limits		IC_{90} (µg/ml)	95% Fiducial limits		Slope ± SE
			Lower	Upper		Lower	Upper	
Central zone								
Khandwa	M.P	0.068[b]	0.036	0.092	0.524[ab]	0.365	0.807	1.412 ± 0.189
Jalna	Maharashtra	0.146[b]	0.068	0.194	0.529[ab]	0.513	1.877	1.571 ± 0.171
Vadodara	Gujarat	0.322[a]	0.193	0.328	1.422[a]	1.082	2.143	1.700 ± 0.164
South zone								
Haveri	Karnataka	0.181[b]	0.129	0.210	0.894[a]	0.665	1.300	1.772 ± 0.177
Rangareddy	AP	0.089[b]	0.041	0.098	0.558[ab]	0.387	0.849	1.431 ± 0.187
Coimbattore	Tamil Nadu	0.077[b]	0.030	0.074	0.221[b]	0.176	0.358	1.978 ± 0.332
SEM		0.05			0.21			
CV %		67.87			67.82			
LSD (P= 0.05)		0.13			0.61			

@ IC_{50} & IC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits

Table 4 : Dose response in growth inhibition (EC) of the Cry2Ab2 surviving *H. armigera* larvae

Location	State	EC_{50} (µg/ml)	95% Fiducial limits		EC_{90} (µg/ml)	95% Fiducial limits		Slope ± SE
			Lower	Upper		Lower	Upper	
Central zone								
Khandwa	M.P	0.033[a]	0.012	0.051	0.234[a]	0.169	0.367	1.453 ± 0.257
Jalna	Maharashtra	0.018[ab]	0.003	0.035	0.288[a]	0.077	0.181	1.547 ± 0.386
Vadodara	Gujarat	0.055[a]	0.015	0.074	0.412[a]	0.195	0.424	1.452 ± 0.245
South zone								
Haveri	Karnataka	0.038[a]	0.013	0.054	0.269[a]	0.204	0.452	1.358 ± 0.224
Rangareddy	AP	0.032[a]	0.010	0.048	0.207[a]	0.152	0.329	1.479 ± 0.276
Coimbattore	Tamil Nadu	0.033[a]	0.005	0.041	0.122[ab]	0.087	0.185	1.775 ± 0.433
SEM		0.009			0.089			
CV %		59.49			78.42			
LSD (P= 0.05)		0.027			0.261			

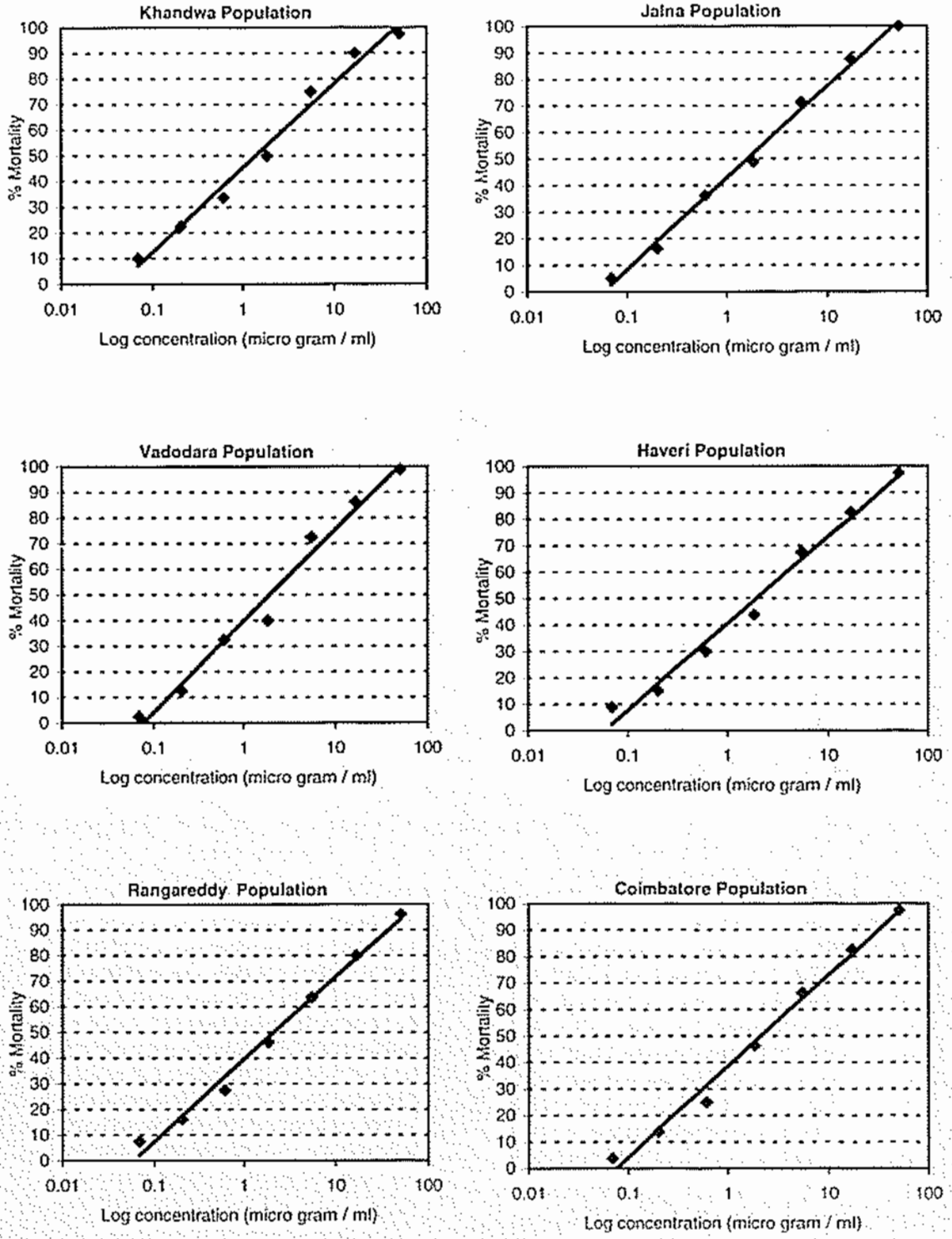
@EC_{50} & EC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits

Table 5. Summary of bioassays with Indian cotton ecosystem field populations of *H. armigera* to Cry2Ab2

S. no.	Bioassay parameter	Six field populations*
1	LC_{50}	1.399 – 2.501 (2.049)
2	LC_{90}	19.777 – 47.809 (29.081)
3	MIC_{50}	0.345 – 0.933 (0.708)
4	MIC_{90}	2.715 – 6.779 (4.444)
5	IC_{50}	0.068 – 0.322 (0.147)
6	IC_{90}	0.221 – 1.422 (0.691)
7	EC_{50}	0.018 – 0.055 (0.035)
8	EC_{90}	0.122 – 0.412 (0.255)

* The above values represent the ranges (µg Cry2Ab2 /ml) with mean in parentheses.

Fig . 1. Log concentration-response lines for mortality of Central and South Indian *H. armigera* populations to Cry2Ab2 protein



Contract research Project
Sponsored by MAHYCO-Monsanto India,
under the directions of the
GEAC, Government of India.

Final Report 2004-2005

Part-1

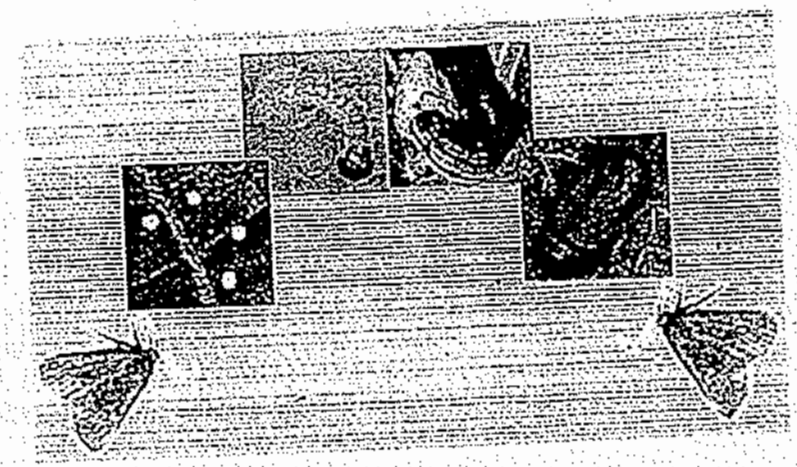
Baseline susceptibility of the cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) to Cry2Ab2 toxin from *Bacillus thuringiensis*

Part –II

Efficacy of Bollgard-II on Cry1Ac resistant *H. armigera*

Part-III

Analysis of Bollgard-II Cotton Seed Oil for *cry2Ab* Gene



Dr K. R. Kranthi
Principal Investigator

Central Institute for Cotton Research
(Indian Council of Agricultural Research)
Post Bag o. 2, Shankarnagar PO, Nagpur 440 010
Phone: 07103-275549, 36. Fax: 07103-275529; e-mail: cicrnpp@rediffmail.com

ICAR

Central Institute for Cotton Research

(Indian Council of Agricultural Research)
Post Bag o. 2, Shankarnagar PO, Nagpur 440 010
Phone: 07103-275549, 36. Fax: 07103-275529; e-mail: cicrngp@rediffmail.com

We hereby certify that the work being reported in the following final report was carried directly under our supervision and that we take full responsibility for the authenticity of the results being reported herein.

Part1. Baseline susceptibility of the cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) to Cry2Ab2 toxin from *Bacillus thuringiensis.* Part-II: Efficacy of Bollgard-II on Cry1Ac resistant *H. armigera* and Part-III: Analysis of Bollgard-II Cotton Seed Oil for *cry2Ab* Gene. 2004-2005

18th August 2005
Nagpur.

Dr K. R. Kranthi
Senior Scientist
Principal Investigator

Certified that the work was carried under the contract research project sponsored by Mahyco-Monsanto Biotech under the directions of the GEAC, Ministry of Environment, Government of India. The results presented in the report were carried out at CICR, Nagpur

18th August 2005
Nagpur.

Dr B. M. Khadi
Director, CICR

Contract Research Project

Final Report 2004-2005

Sponsored by MAHYCO-Monsanto India, under the directions of the GEAC, Government of India.

Dr K. R. Kranthi
Principal Investigator

Central Institute for Cotton Research,
PB.No 2, Shankarnagar PO, Nagpur, 440 010.
Phone: Office: 07103-275549(ext 302)
Fax: Office: 07103-275529

Part-1

Baseline susceptibility of the cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) to Cry2Ab2 toxin from *Bacillus thuringiensis*

Abstract

The geographical variability in *H. armigera* susceptibility levels to Cry2Ab2 toxin from *Bacillus thuringiensis*, was determined through log dose probit assays conducted on populations collected from 25 cotton-growing districts across India. The LC_{50} values ranged from 6.0 to 28.6 µg Cry2Ab2/ml of diet with 4.8-fold variability in susceptibility across the strains. The IC_{50} range indicated 7-fold variability with the values ranging from 0.31 to 2.3 µg/ml. The cumulative LC_{50} and IC_{50} values for the entire data sets were 15.21 and 0.71 µg/ml respectively. The data were used to derive the LC_{99} and IC_{99} values of 7577 and 37.72 µg/ml respectively, which can be used as diagnostic concentrations to monitor the increase in resistant individuals in field populations. The probit analysis data can be used as baseline indices to monitor for changes in the *H. armigera* susceptibility to Cry2Ab2, subsequent to the introduction of Bollgard-II cotton in India.

Introduction

Cry2Ab2 is a crystal protein derived from the soil bacterium *Bacillus thuringiensis* (Bt). It is toxic to lepidopteran insects including the cotton bollworm *Helicoverpa armigera*[1], which is one of the most important economic insect pests in many parts of the world including Asia, Australia and Africa. Recently, the combination of *cry2Ab2* and *cry1Ac* genes in Bollgard-II cotton has been permitted for commercial use in USA. Bollgard-I, which expresses a single gene *cry1Ac* has been in cultivation in the USA for almost a decade and is now considered as one of the best technological advances for cotton pest management. However, the deployment of single genes for the expression of insecticidal toxin proteins in crop plants is expected to select for resistance in the target pests over a period of continuous exposure. The capacity of *H. armigera* to develop

resistance to Cry1Ac has been demonstrated by laboratory selection in Australia[1], China[2] and India[3]. Ecological modeling[4] shows that the use of two genes specifying two different insecticidal proteins in the same plant, as opposed to the use of a single gene, as in Bollgard-I, is likely to delay the selection of insects resistant to the insecticidal proteins by a factor of 10. Thus, simultaneous deployment of two or more toxins that are not cross-resisted by the target pest, is considered to be one of the most useful strategies of resistance management. Competition studies[5] indicated that Cry1A and Cry2A toxins bind to different receptors in target insects and are not cross-resisted by *H. armigera*[1]. Due to the difference in structure and insecticidal mechanism, the *cry2A* genes are promising candidates for management of resistance in insects[6].

Bollgard-II, is being tested in large scale field trials in India and is likely to be released for commercial cultivation within a couple of years. It was found to have superior levels of insecticidal activity compared to Bollgard-I and in particular to augment the late season insect control[7, 8]. The dual gene technology is being considered as an improvised pest management method not just for its enhanced efficacy, but also as an efficient resistance management strategy. For resistance management programmes to be effective, monitoring, surveillance and early detection of resistance are important prerequisites. Regular monitoring for resistance development helps to detect the emergence of resistant phenotypes in order to initiate timely remedial measures[9]. Thus, it is important that resistance development to the new toxin in the target pest is monitored carefully so that it is not allowed to increase to levels that impair its efficacy. Baseline susceptibility of *H. armigera* to Cry1Ac in India is known[10-12], but there are no data on the extent of variability in *H. armigera* response to Cry2Ab2. The current study aims to understand the geographical variability of baseline susceptibility in the cotton bollworm, *H. armigera* to the Cry2Ab2 toxin in India, especially before the introduction of Bollgard-II for commercial cultivation.

Materials and Methods

Laboratory strains of *H. armigera* were established from larvae collected in cotton fields during the cropping season of 2004-2005 from major cotton growing regions India. Field strains of the cotton bollworm *H. armigera* were collected during October-December 2004, on cotton fields from 11 districts of central India (Nagpur, Hingoli, Aurangabad, Parbhani, Wardha, Chindwara, Bharuch, Vadodara, Surat, Surendranagar and Amreli), 8 districts of North India (Hanumangarh, Sirsa, Fatehabad, Sriganganagar, Abohar, Mansa, Hisar and Bhatinda) and 6 districts of South India (Warangal, Khammam, Karimnagar, Guntur, Dharwad and Coimbatore). The strains were established on semisynthetic diet. Larvae were reared on a chickpea based semisynthetic diet[13] individually in 7.5 ml cells of 12 well 'ICN-Linbro' tissue culture plates until pupation. Moths were kept in glass jars and fed on 10 % honey solution. A layer of muslin cloth was placed on the inner surface of the jar for oviposition. One-day old larvae were tested at the rate of one per well at a total of twenty to twenty four larvae per concentration on semi-synthetic diet incorporating different concentrations of the toxin. The Cry2Ab2 protein was provided by Monsanto, India, as Bt-corn leaf powder that contained 3.936 mg Cry2Ab2 per g of the leaf powder. A total of 5 concentrations of the toxins ranging from 0.078 to 19.68 µg Cry2Ab/ml diet were used for the bioassays.

Mortality was recorded daily until the sixth day. Weight of the surviving larvae was recorded on the final day of observation. The assays were performed in the laboratory at conditions of 27 $\pm 1^0$C and 70% relative humidity. Median Lethal Concentrations (LC_{50}) presented in table 1, and median inhibitory concentrations (IC_{50}) presented in table 2, were derived from log dose probit calculations[14]. IC_{50} values represent the median inhibitory concentrations that prevent 50% of individuals in the treated population from reaching half the average weight of control larvae.

Results and Discussion

The bioassay results showed that, compared to Cry1Ac, the Cry2Ab2 toxin was found to be at least 10-fold less toxic to *H. armigera*. The geographical variability in *H. armigera* susceptibility levels to Cry2Ab2 was minimum. The LC_{50} values ranged from 6.0 to 28.6 µg Cry2Ab2/ml of diet. The range of LC_{50} was 9.71 to 17.84 in north India, 10.19 to 28.60 in central India and 6.0 to 17.96 µg/ml of diet in south India. The variability in susceptibility across the strains was 4.8 fold. The most susceptible LC_{50} value of 6.0 µg/ml was observed in populations collected from Khammam district in Andhra Pradesh and the highest value of 28.60 µg/ml from Aurangabad in Central India. The IC_{50} range indicated 7-fold variability in *H. armigera* response to Cry2Ab2. The populations collected from Bharuch in central India exhibited the lowest value of 0.31 µg/ml, whereas the highest IC_{50} value of 2.30 µg/ml was observed in samples collected from Bhatinda in north India. The fiducial limits (FL) at 95% probability, and the χ^2 values of the probit assay data indicated that the variability in response of the different *H. armigera* populations to Cry2Ab2 was minimum. This is in contrast to the response of *H. armigera* populations to Cry1Ac, in which heterogeneity was high in most of the field strains tested[10]. The bioassay data obtained from all the strains were subjected to probit analysis to obtain the cumulative LC_{50} (95% FL) and IC_{50} (95% FL) values of 15.21 (12.51 - 18.95) and 0.74 (0.65 - 0.83) µg/ml respectively. The data were also used to derive the LC_{99} (95% FL) and IC_{99} (95% FL) values of 7577 (3934 – 16543) and 37.72 (28.08 – 53.05) µg/ml respectively. Theoretically, the values can be used as diagnostic concentrations to monitor the increase in resistant individuals in field populations. But, a Cry2Ab2 concentration of 7.577 g/l diet is impossible to achieve using Bt-corn leaf powder (0.39% Cry2Ab2) and would be extremely expensive if the toxins were to be produced from Cry2Ab expressing *E. coli* clones. However, the Bt-corn leaf powder can be incorporated at 9.58 g into 1 l semi-synthetic diet to obtain a toxin based diet with a concentration of 0.037 g/l to monitor the evolution of resistance based on the IC_{99} as a growth inhibitory diagnostic concentration.

The low range of variability in the baseline data of *H. armigera* susceptibility to Cry2Ab2 is not surprising. The *cry2Ab2* gene occurs naturally in *Bacillus thuringiensis* var. *Kurstaki*, but is either not expressed or has low expression due to an inefficient promoter[15]. It is therefore likely that the toxin would not have been present in adequate quantities in the Bt formulations that were used for pest control in India, to cause variability in the baseline susceptibility of *H. armigera* to Cry2Ab2. However, it is possible that the related toxin Cry2Aa, which is 88% identical to Cry2Ab2, and is present in the Bt formulations, may have been responsible for whatever little variation that may have been observed between the populations across India.

The LC_{50} values indicate that it is unlikely that Cry2Ab2 alone can be used for the control of *H. armigera*. There are very few published studies on the toxicity of Cry2Ab2 to *H. armigera*. Cry2Ab was reported sublethal[6], but Cry2Aa was reported to be 30-fold less toxic than Cry1Ac, to *H. armigera*[16-18]. Bollgard-II cotton which expresses both Cry1Ac and Cry2Ab2 was developed to enhance the bollworm control efficacy of the single gene *cry1Ac* based Bollgard-I. Interestingly the expression levels of Cry2Ab2 in Bollgard-II are at least 10 fold higher than Cry1Ac[19]. The higher expression levels compensate for the lower insecticidal activity of Cry2Ab2 against bollworms, especially *H. armigera*. Cry1Ac and Cry2Ab2 are two of a diverse family of insecticidal proteins expressed by *Bacillus thuringiensis*. The proteins are grouped in classes that exhibit different specificities to different lepidopteran caterpillars[20, 15, 21]. Bollgard-II was reported[8, 22] to be superior in its toxicity to cotton bollworms and a range of lepidopteran insect pests including the armyworm, *Spodoptera* spp, which are otherwise not effectively controlled by Bollgard-I.

Apart from conferring enhanced efficacy against bollworms, as an additive toxin to Cry1Ac, the Cry2Ab2 toxin is extremely useful for resistance management. The two toxins (Cry1Ac and Cry2Ab2) share less than 20% homology and thus have resistance mechanisms that are strikingly different[5]. Binding of Bt toxins to gut receptors lead to pore formation in the cell membrane[23]. The pores formed by Cry2Aa, a toxin closely related to Cry2Aab, differ from those formed by Cry1Ac[24], thereby suggesting a mechanistic difference in insecticidal activity between the two toxins. Moreover, Cry1Ac resistant *H. armigera* strains exhibit susceptibility to Cry2Ab, thus indicating the absence of cross-resistance[1]. Therefore even if the target pest develops resistance to one of the toxins, it is likely that the other toxin will continue to be effective, thereby limiting the potential for the resistant individual to pass the trait on to subsequent generations. However, since Cry1Ac expression declines remarkably towards the end of the season[25] and Cry2Ab2 levels are at 10-fold higher than the Cry1Ac, there would be a possible increased risk of insect pests evolving resistance to the Cry2Ab2 toxin alone. It is pertinent to point out here that, since Cry2Ab2 is inherently less toxic to *H. armigera*, even low resistance levels to the toxin in the insect, can render the Cry2Ab2 less effective, thus impairing the selective advantage of Bollgard-II over Bollgard-I. It is therefore necessary to initiate proactive resistance management strategies to delay the evolution of resistance against either of the two toxins to ensure sustained efficacy of the dual gene Bt-cotton. The current baseline data enable monitoring changes in *H. armigera* susceptibility to Cry2Ab2 and also the concomitant changes in the pest management efficacy of the dual-gene Bt-transgenic plants in India consequent to the introduction of Bollgard-II.

Acknowledgements

The work was carried out under the directives of GEAC with funding support from Monsanto-Mahyco Biotech, India. We thank Monsanto, India, for kindly providing us the Cry2Ab2 toxin.

References:

1. Akhurst, R. J., James, W., Bird, L. J. and Beard, C. Resistance to the Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in the cotton bollworm *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, 2003, 96, 1290-1299.
2. Liang, G., Tan, W. and Guo, Y. Study on screening and mode of inheritance to Bt transgenic cotton in *Helicoverpa armigera*. *Acta Entomologia sinica*, 2000, 43, 57-62.
3. Kranthi, K. R., Kranthi, S., S. Ali. and Banerjee, S. K. Resistance to Cry1Ac δ-endotoxin of *Bacillus thruringiensis* in a laboratory selected strain of *Helicoverpa armigera* (Hübner). *Current Science*, 2000, 78, 1001-1004.
4. Roush, R. Two toxin strategies for management of insecticidal transgenic crops: Can pyramiding succeed where pesticide mixtures have not? *Phil. Trans. Royal Soc. Lond. B*, 1998, 353, 1777 –1786.
5. Morse, R. J., Yamamoto, T. and Stroud, R. M. Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure Folding Design*, 2001, 9, 409-417.
6. Shantanu Kumar., Udayasuriyan, V., Sangeetha, P. and Bharathi, M. Analysis of Cry2A proteins encoded by genes from indigenous isolates of *Bacillus thuringiensis* for toxicity against *Helicoverpa armigera*. *Current Science*, 2004, 86, 566-570.
7. Jackson, R. E., Bradley, J. R., Burd, A. D. and Van Duyn, J. W. Field and greenhouse performance of bollworm on Bollgard-II genotypes. In: *Proceedings of the Beltwide Cotton Conference*. Dugger, P. A and Richter D. (Eds). National Cotton Council, Memphis, USA. 2002,1048-1051.
8. Akin, D. S., Stewart, S. D. and Knighten, K. S. Field efficacy of cotton expressing two insecticidal proteins of *Bacillus thuringiensis*. In: *Proceedings of the Beltwide Cotton Conference*. Dugger, P. A and Richter D. (Eds). National Cotton Council, Memphis, USA. 2001, 2, 1041-1043.
9. Sims, S. B., Greenplate, J. T., Stone, T. B., Caprio, M. A. and Gould, F. L. Monitoring strategies for early detection of Lepidoptera resistance to *Bacillus thuringiensis* insecticidal proteins, pp. 229-242. *In* Brown, T. M (ed.) *Molecular genetics and Evolution of pesticide resistance*. ACS Symposium Series No. 645. American Society Washington. DC. 1996.
10. Kranthi, K. R., Kranthi, S and Wanjari, R. R. Baseline toxicity of Cry1A toxins to *Helicoverpa armigera*. *International Journal of Pest Management*, 2001, 47, 141-145.
11. Gujar, G. T., Kumari, A., Kalia, V. and Chandrasekhar, K. Spatial and temporal variation in susceptibility of Americal bollworn, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* var. *Kurstaki*. *Current Science*, 2000, 78, 995-1001.
12. Jalali, S. K., Mohan, K. S., Singh, S. P., Manjunath, T. M. and Lalitha, Y. Baseline susceptibility of the old-world bollworm, *Helicoverpa armigera* (Hübner), (Lepidoptera: Noctuidae) populations from India to *Bacillus thuringiensis* Cry1Ac insecticidal protein. *Crop Protection*, 2004, 23, 53-59.

13. Armes, N. J., Bond, G. S. and Cooter, R. J., *The laboratory culture and development of Helicoverpa armigera.*, Natural Resources Institute Bulletin 57, Natural Resources Institute, Chatham, UK, 1992.
14. Finney, D. J. Probit analysis. 3rd edition Cambridge University Press. 1971.
15. Dankocsik, C., Donovan, W. P. and Jany, C. S. Activation of a cryptic crystal protein gene of *Bacillus thuringiensis* subsp. *Kurstaki* by gene fusion and determination of the crystal protein insecticidal specificity. *Molecular Microbiology*, 1990, 4, 2087-2094.
16. Chakrabarthi, S. K., Mandaokar, A., Kumar, P. A. and Sharma, R. P. Efficacy of lepidopteran specific δ-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera*. *Journal of Invertebrate Pathology*, 1998, 72, 336-337.
17. Babu, B. G., Udayasuriyan, V., Asia Mariam, M., Sivakumar, N., Bharathi, M. and Balasubramanian, G. Comparative toxicity of Cry1Ac and Cry2Aa δendotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera*. *Crop Protection*, 2002, 21, 817-822.
18. Lenin, K., Asia Mariam, M. and Udayasuriyan, V. Expression of a *cry2Aa* gene in an acrstalliferous *Bacillus thuringiensis* strain and toxicity of Cry2Aa against *Helicoverpa armigera*. *World Journal of Microbiology & Biotechnology*. 2001, 17, 273-278.
19. Greenplate, J. T., Penn, S. R., Mullins, J. W. and Oppenhuizen, M. Seasonal Cry1Ac levels in DP50B: The "Bollgard® basis" for Bollgard II. In: *Proceedings of the Beltwide Cotton Conference*. Dugger, P. A and Richter D. (Eds). National Cotton Council, Memphis, USA. 2000, 2, 1039-1040.
20. Macintosh, S. C., Stone, T. B., Sims, S. R., Hunst, P. L., Greenplate, J. T., Marrone, P. G., Perlak, F. J., Fischhoff, D. A. and Fuchs, R. L. Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. *Journal of Invertebrate Pathology*, 1990, 56: 258-266.
21. Widner W. R. and Whiteley, H. R. Two highly related crystal proteins of *Bacillus thuringiensis* subsp. *Kurstaki* possess different host range specificities. *Journal of Bacteriology*. 1989, 171, 965-974
22. Gore, J., Leonard, B. R. and Adamczyk, J. J. Bollworm (Lepidoptera: Noctuidae) Survival and 'Bollgard II' Cotton Flower Bud and Flower Components. *Journal of Economic Entomology*, 2001, 94, 1445-1451.
23. Knowles, B. H. and Dow, J. A. T. The crystal delta-endotoxins of *Bacillus thuringiensis*: models for their mechanism of action on the insect gut. *Bioassays* 1993, 15, 469-476.
24. English, L., Robbins, H. L., Von Tersch, M. A., Kulesza, C. A., Ave, D., Coyle, D., Jany, C. S. and Slatin, S. L. Mode of action of CryIIA: a *Bacillus thuringiensis* delta-endotoxin. *Insect Biochemistry and Molecular Biology*, 1994, 24, 1025-1035.
25. Kranthi, K. R., Naidu, S., Dhawad, C. S., Tatwawadi, A., Mate, K., Patil, E., Bharose, A. A., Behere, G. T., Wadaskar, R. M. and Kranthi, S. Temporal and intra-plant variability of Cry1Ac expression in Bt-cotton and its influence on the survival of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera). *Current Science*, 2005, 88, (In press).

Table 1. Median lethal (LC_{50}) baseline susceptibility of *H. armigera* to Cry2Ab2 in field populations collected from twenty districts of India during 2004-05.

District	n	LC_{50}	95% FL	Slope ± S.E	χ^2
South					
Guntur	120	11.59	6.00 – 35.29	1.05 ± 0.20	0.89
Warangal	120	15.24	7.48 – 57.04	1.02 ± 0.22	0.52
Karimnagar	120	8.26	4.48 – 20.86	1.08 ± 0.21	0.72
Khammam	120	6.00	3.42 – 12.94	1.13 ± 0.20	0.61
Dharwad	120	17.96	8.29 – 83.06	0.96 ± 0.22	0.58
Coimbatore	120	8.91	4.62 – 25.40	0.98 ± 0.18	1.04
North					
Abohar	120	17.84	7.93 -90.26	0.91 ± 0.21	0.92
Hissar	120	11.92	6.94 -32.01	1.19 ± 0.22	0.39
Fatehabad	120	16.04	7.30 -73.83	0.92 ± 0.20	0.77
Sriganganagar	120	14.92	6.53 -75.07	0.85 ± 0.19	2.57
Sirsa	120	14.45	6.75 -60.01	0.93 ± 0.20	0.61
Hanumangarh	120	13.59	6.58 – 50.41	0.97 ± 0.20	1.00
Mansa	120	11.98	5.56 -48.39	0.87 ± 0.19	1.36
Bhatinda	120	9.71	4.53 -36.94	0.84 ± 0.18	2.50
Central					
Bharuch	100	12.30	5.31 – 65.07	0.86 ± 0.21	1.13
Vadodara	120	11.90	6.54 – 31.75	1.19 ± 0.24	0.42
Surat	100	22.55	8.89 – 205.6	0.90 ± 0.23	0.49
Surendranagar	120	13.89	6.96 – 48.02	1.04 ± 0.22	0.46
Amreli	120	18.50	7.73 -114.51	0.84 ± 0.19	1.03
Wardha	120	18.44	7.74 – 112.64	0.85 ± 0.19	1.12
Aurangabad	120	28.60	12.47 -206.60	1.05 ± 0.27	1.37
Chindawara	120	20.82	8.44 -147.08	0.83 ± 0.19	0.86
Hingoli	120	12.69	6.48 – 40.80	1.04 ± 0.22	0.67
Parbhani	120	10.19	4.68 – 41.27	0.82 ± 0.18	2.03
Nagpur	120	16.24	7.69 – 67.93	0.98 ± 0.21	0.66

LC_{50} is expressed in µg/g diet; n = the number of test insects; S.E = Standard Error; FL = Fiducial Limits.

Table 2. Median inhibitory (IC_{50}) baseline susceptibility of *H. armigera* to Cry2Ab2 in field populations collected from twenty districts of India during 2004-05.

District	n	IC_{50}	95% FL	Slope ± S.E	χ^2
South					
Guntur	120	1.26	0.66 - 2.37	1.68 ± 0.36	1.64
Warangal	120	1.57	0.81 - 3.07	1.58 ± 0.34	2.03
Karimnagar	120	0.59	0.34 – 0.98	1.25 ± 0.19	1.36
Khammam	120	0.45	0.27 – 0.72	1.47 ± 0.20	0.41
Dharwad	120	0.92	0.59 – 1.42	1.63 ± 0.21	0.69
Coimbatore	120	0.69	0.42 – 1.10	1.41 ± 0.21	1.32
North					
Abohar	120	0.83	0.17-3.12	1.43 ± 0.22	5.98
Hissar	120	0.84	0.34-1.88	1.60 ± 0.24	3.30
Fatehabad	120	1.10	0.73-1.66	1.85 ± 0.28	1.28
Sriganganagar	120	0.75	0.49-1.12	1.89 ± 0.29	1.38
Sirsa	120	0.88	0.36-1.98	1.59 ± 0.24	3.27
Hanumangarh	120	1.04	0.66-1.61	1.63 ± 0.25	2.98
Mansa	120	2.05	0.75-6.37	1.58 ± 0.24	4.46
Bhatinda	120	2.30	0.83-8.07	1.43 ± 0.22	7.99
Central					
Bharuch	100	0.31	0.18-0.50	1.67 ± 0.31	2.14
Vadodara	120	0.45	0.14-1.05	1.66 ± 0.26	7.06
Surat	100	0.45	0.16-1.02	1.93 ± 0.34	3.26
Surendranagar	120	1.05	0.67-1.63	1.62 ± 0.25	2.80
Amreli	120	0.67	0.26-1.50	1.68 ± 0.26	3.48
Wardha	120	0.79	0.43-1.43	1.96 ± 0.44	0.51
Aurangabad	120	0.50	0.19-1.09	1.65 ± 0.26	6.05
Chindawara	120	1.11	0.73-1.69	1.76 ± 0.27	1.46
Hingoli	120	0.97	0.59-1.63	3.07 ± 0.83	0.36
Parbhani	120	1.31	0.38-4.52	1.44 ± 0.22	5.10
Nagpur	120	1.24	0.63-2.42	1.55 ± 0.33	1.64

IC_{50} is expressed in µg/g diet; n = the number of test insects; S.E = Standard Error; FL = Fiducial Limits.

Part-II

Efficacy of Bollgard-II on Cry1Ac resistant *H. armigera*

Introduction

The cotton bollworm *Helicoverpa armigera* has a demonstrated capability to develop resistance to Cry1Ac toxin that was derived from *Bacillus thuringiensis*. The current Bt-cotton crop commercially released in India, deploys *cry1Ac* as the transgene to protect itself from cotton bollworms including *Helicoverpa armigera*. Continuous exposure of H. armigera to Bt-cotton, would allow resistant insects to survive and proliferate, thereby leading to resistance under field conditions. The use of two or more toxins as mixtures is believed to enhance toxicity and also delay resistance to both toxins. However it is important that the toxins are unrelated to each other in terms of their mode of action and resistance mechanisms in the target insect. Ecological modeling clearly shows that two unrelated toxins in the same plant have the potential to delay resistance at least by a factor of 10. Thus mixtures are considered as one of the most promising resistance management strategies. Recently, the combination of *cry2Ab2* and *cry1Ac* genes in Bollgard-II cotton has been permitted for commercial use in USA. Due to the difference in structure and insecticidal mechanism the Cry1A and Cry2A toxins are known to bind to different receptors in target insects.

The objectives of this study were 1. Determine the comparative toxicity of Cry2Ab on Cry1Ac resistant and susceptible *H. armigera* larvae 2. Assess the toxicity of 'Cry1Ac+Cry2Ab' mixture on Cry1Ac resistant and susceptible *H. armigera* larvae, and 3. Examine the relative survival rate of Cry1Ac resistance and susceptible H. armigera larvae on non-Bt cotton, Bollgard-I and Bollgard-II.

Materials and Methods

The susceptible strain SUS-G was isolated from F_2 progeny of single pair mated isofemale moths from Bt susceptible populations using methods described by Andow and Alstad, 1998. The strains were maintained in the laboratory on a wheatgerm based semi-synthetic diet. The resistant strain RES-Bt-a was derived from a field population collected as survivors on Bt cotton. It was selected for two generations on plant parts of Bt-cotton (Var: MECH-162-Bt) and the next subsequent 6 generations on semi-synthetic diet either layered or incorporated with diluted stock solutions of MVP-II, (Dow Agrosciences San Diego, CA) a liquid formulation containing 19.7% Cry1Ac encapsulated in *Pseudomonas fluorescens*. The Cry1Ac in MVP-II is 99% identical to the active toxin region of the Cry1Ac expressed in Bt cotton. The Cry2Ab2 protein was provided by Monsanto, India, as Bt-corn leaf powder that contained 3.936 mg Cry2Ab2 per g of the leaf powder. One-day old larvae were tested at the rate of one per well at a total of twenty to twenty four larvae per concentration on semi-synthetic diet incorporating at least four different concentrations of the toxin. For the bioassays using a combination of toxins, Cry2Ab2 was added @ 20 µg Cry2Ab2/ml of diet each to the 5 different concentrations of Cry1Ac used in the range of 0.01 to 5.0 µg Cry2Ab2/ml of diet. Mortality was recorded daily until the sixth day. The assays were performed in the

laboratory at conditions of 27 $\pm 1^0$C and 70% relative humidity. Median Lethal concentration (LC_{50}) values and their 95% fiducial limits (FL) presented in table 1 were derived from log dose calculations computed by probit analysis. When required, corrections for control mortality were made using Abbott's formula. The Cry1Ac resistant 'RES-Bta' and susceptible 'SUS-G' larvae were released on terminal leaves of Bt cotton 'MECH-162-Bollgard-1', 'MECH-162-Bollgard-II' and non-Bt MECH-162 (60-70 days after sowing) individually in perforated plastic cups. The leaves were changed daily and mortality observations were recorded after 6 days. Ten plants each of the Bt cotton 'MECH-162-Bollgard-1', 'MECH-162-Bollgard-II' and non-Bt MECH-162 were grown in the green house. Five neonates were released per plant. Observations were recorded after six days.

Results and Discussion

The resistant strain RES-Bt-a was found to be 82-fold resistant to Cry1Ac as compared to the susceptible SUS-G strain. The bioassays with Cry2Ab showed that the resistant strain RES-Bt-a was as susceptible as the susceptible strain SUS-G, as indicated by the overlap of fiducial limits. Cry2Ab was found to cause a mortality of 40-50% in the Cry1Ac susceptible strains, at a concentration of 20 µg toxin incorporated into semi-synthetic diet. Bioassays were carried out with a toxin mixture combination containing variable concentrations of Cry1Ac added with 20 µg Cry2Ab. Results showed that the combination was highly potent with a capability to cause high levels of mortality in Cry1Ac resistant *H. armigera*. The LC_{50} values with the mixture in the SUS-G strain were lower than that of the values obtained with Cry1Ac on susceptible *H. armigera* strains, indicating an additive effect of the Cry2Ab. The LC_{50} values for Cry1Ac in the RES-Bt-a strain, with the mixture were 26-fold lower than that of the values obtained with Cry1Ac alone in the RES-Bt-a strain. The data clearly showed that Cry2Ab is toxic to the Cry1Ac resistant *H. armigera*, and can thus contribute significantly to resistance management. The Cry1Ac resistant strain showed a survival of 56.2% on Bollgard-I, as compared to 91.7% on Bollgard-II. The Cry1Ac resistant larvae that survived on Bollgard-II, were found to be severely stunted, with a 60-82% reduction in weight as compared to the larvae on non-Bt cotton. The green house tests were not very informative. More than 75% larvae were missing on the non-Bt plants. It is not clear, as to what would have happened to the larvae. The surviving larvae were found to have reached the third-fourth instar stage on non-Bt plants. Out of the 25 Cry1Ac resistant larvae released, only two survived on bollgard-I and one survived on the bollgard-II plants. However these larvae were found to be severely stunted. Only one larva from the 25 Cry1Ac susceptible larvae survived the Bollgard-I plants. The studies clearly showed that the Cry1Ac and Cry2Ab when used in combination had superior levels of insecticidal activity compared to each of the toxins used alone. Thus, the dual gene technology can be considered not just as an improvised pest management method but also as technology with a strong potential to delay resistance as well.

Table 1. Susceptibility to Cry1Ac (MVP-II)

Strain	n	LC_{50} (95% FL)	LC_{90} (95% FL)	Slope ± SE	RF
RES-Bt-a	288	7.43 (4.3-20.4)	68 (24-620)	1.3 ± 0.2	82
SUS-G	288	0.09 (0.07-0.12)	0.36 (0.26-0.55)	2.2 ± 0.2	

Table 2. Susceptibility to Cry2Ab

Strain	n	LC_{50} (95% FL)	LC_{90} (95% FL)	Slope ± SE	χ^2
RES-Bt-a	120	32.8 (12.6-364)	837 (125-24519)	0.9 ± 0.2	2.54
SUS-G	120	23.7 (9.6-179)	719 (115-10524)	0.9 ± 0.2	1.24

Table 3. Susceptibility to *Cry1Ac (mixtures containing variable concentrations of Cry1Ac added with 20 µg Cry2Ab)

Strain	n	LC_{50} (95% FL)	LC_{90} (95% FL)	Slope ± SE	χ^2
RES-Bt-a	240	0.28 (0.12-0.56)	54.17 (11.3 –2112)	0.6 ± 0.1	1.06
SUS-G	288	0.02 (0.008-0.028)	0.11 (0.06-0.292)	1.6 ± 0.2	4.19

*LC_{50} values derived from the Cry1Ac raw data

Table 4. Susceptibility of Cry1Ac susceptible and resistant *H. armigera* strains to leaves of Bollgard-I and Bollgard-II cotton hybrids. *Invitro* bioassay

	RES-Bt-a		SUS-G	
	d/n	% mortality	d/n	% mortality
MECH-162-Bollgard-II	44/48	91.7	48/48	100
MECH-162-Bollgard-1	27/48	56.2	46/48	95.8
MECH-162	0/24	0	0/24	0

d=dead; n= number tested

Table 5. Susceptibility of Cry1Ac susceptible and resistant *H. armigera* strains to Bollgard-I and Bollgard-II cotton hybrids. Greenhouse release.

	RES-Bt-a		SUS-G	
	A/n	% Survival	A/n	% Survival
MECH-162-Bollgard-II	1/25	4	0/25	0
MECH-162-Bollgard-1	2/25	8	1/25	4
MECH-162	6/25	24	5/25	20

A=alive; n= number tested

Part-III

Analysis of Bollgard-II Cotton Seed Oil for *cry2Ab* Gene

Objective:

To detect the presence of *cry2Ab* gene in the oil extracted from seeds of Bollgard-II

Materials and methods:

Seeds of Bollgard-II (MECH-162 BG-II) and Non-Bt MECH-162, were acid delinted and crushed in hexane. The homogenate was filtered through 3-4 layers of muslin cloth to extract the raw oil. Hexane was evaporated in fumehood to obtain raw oil. The oil was centrifuged at 35,000 x g to remove debris of seed coat and other particles.

DNA was isolated from tender leaves of Bollgard-II (MECH-162 BG-II), to be used as internal checks along with the oil sample. DNA was isolated from the oil using the following method. 500 µl of oil was mixed thoroughly with 500 µl of lysis buffer in a 2ml plastic vial and heated at 60^{0}C for 30 minutes. The mixture was centrifuged and the aqueos phase was transferred into a fresh vial and 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added. The mixture was mixed well and centrifuged at 10,000 x g for 10 min. The aqueous phase was re-extracted with chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 x g for 10 min. The supernatant was taken in a fresh tube and DNA was precipitated with equal volume of isopropanol. The tubes were kept at –20^{0}C for 30 minutes and centrifuged at 12,000 x g for 10 min to obtain the DNA pellet. The supernant was decanted and the pellet was washed once with 70% ethanol. The resultant pellet was air dried and dissolved in 25 µl of sterile water. The process was repeated separately with oil samples into which 500 ng DNA isolated from leaves of Bollgard-II were added. The DNA thus isolated was subjected to PCR using cry2Ab specific primers. The DNA samples were heated at 95^{0}C for 5 minutes to inactivate any possible nucleases in the samples.

The tubes were subsequently chilled on ice. 5 µl of these samples was used as template for PCR. In addition, each reaction tube contained the following: 2.0 µl 10x Taq buffer; 5 pico moles of each of the forward and reverse primers specific for internal sequence of the *cry2Ab* gene, 200 µM of dNTPs, 1.0 µl Taq DNA polymerase in a total volume of 20 µl. The PCR conditions were 94^{0}C –3 minutes, 94^{0}C –30 seconds, 57^{0}C – 30 seconds, 72^{0}C –90 seconds, 39 cycles of steps 2-4, 72^{0}C –8 minutes and 4^{0}C –until sample recovery. 20 ul of the PCR product was mixed with 2 ul of 10 X gel-loading buffer (50 % glycerol in dd H_2O, 0.1 % bromophenol blue) and 12 ul was electrophoresed on a 1.0 % agarose gel (TBE) at 50V.

DNA bands were stained with ethidium bromide and photographed on a UV transilluminator using KODAK EDAS 290.

ANNEXURE 6.5.2

Insect resistance management plan for Bollgard II™ Cotton In India

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Crop Protection 23 (2004) 53–59
Crop Protection
www.elsevier.com/locate/cropro

Baseline-susceptibility of the old-world bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) populations from India to *Bacillus thuringiensis* Cry1Ac insecticidal protein

S.K. Jalali[a], K.S. Mohan[b,*], S.P. Singh[a], T.M. Manjunath[b], Y. Lalitha[a]

[a] *Project Directorate of Biological Control, Post Bag No. 2491, H. A. Farm Post Bellary Road, Bangalore 560 024, India*
[b] *Monsanto Research Centre, 4412A Bellary Road, N.H. 7, Hebbal, Bangalore 560 092, India*

Received 8 January 2003; received in revised form 17 July 2003; accepted 18 July 2003

Abstract

The baseline-susceptibility of Indian populations of *Helicoverpa armigera* (Hübner) to the insecticidal protein Cry1Ac, from *Bacillus thuringiensis* (Berliner) was determined through bioassays conducted in 1999 and 2001. Populations of *H. armigera* were collected from cotton fields of nine major cotton growing states in India, which included (field locations in parentheses) Punjab (Bathinda), Haryana (Sirsa), Rajasthan (Sriganganagar), Madhya Pradesh (Barwah and Khandwa), Gujarat (Rajkot, Vadodra and Anand), Maharashtra (Jalgoan, Jalna, Akola and Yavatmal), Andhra Pradesh (Adilabad, Warangal, Khammam and Guntur), Karnataka (Raichur, Davangere and Ranebennur) and Tamil Nadu (Coimbatore and Dindigul). All populations were susceptible to Cry1Ac. The mean lethal concentrations, LC_{50}—ranged from 0.14 to 0.71 and from 0.11 to 0.61; LC_{90} ranged from 1.17 to 6.94 and from 1.02 to 6.70 μg of Cry1Ac / ml of diet in 1999 and 2001 populations, respectively. Similarly, moult inhibitory concentrations, MIC_{50}—ranged from 0.05 to 0.27 and from 0.05 to 0.14; MIC_{90}—values ranged from 0.33 to 1.58 and from 0.25 to 0.91 μg of Cry1Ac in 1999 and 2001 populations, respectively. The effective concentrations (weight stunting related) EC_{50} ranged from 0.003 to 0.008 and from 0.0003 to 0.004; EC_{90} ranged from 0.029 to 0.076 and from 0.009 to 0.054 μg of Cry1Ac in 1999 and 2001 populations, respectively. These values form the baseline data for susceptibility of *H. armigera* to Cry1Ac and can be used as benchmarks for monitoring resistance to Cry1Ac. *Bt* cotton, expressing Cry1Ac, was approved for commercial cultivation in India in 2002.
© 2003 Elsevier Ltd. All rights reserved.

Keywords: *Bacillus thuringiensis*; Baseline-susceptibility; Cry1Ac; *Helicoverpa armigera*; Indian population

1. Introduction

Insect pests are primarily responsible for the low yield of cotton in India. Cotton is inhabited by 162 insect species of which a dozen or so are economically important causing considerable yield reduction (Sundaramurthy, 1986; Dhawan et al., 1988; Satpute et al., 1988). The seriousness of the pest problem can be judged from the fact that cotton alone receives 54% of the total insecticides used in plant protection in India. The cotton bollworm complex in India includes the old-world bollworm, *Helicoverpa armigera* (Hübner); spotted bollworm, *Earias vittella* (Fabricius); spiny bollworm, *E. insulana* (Boisduval) and pink bollworm, *Pectinophora gossypiella* (Saunders). Among the bollworms, *H. armigera* is the most important and difficult to control and crop losses in India due to this pest are estimated to be US $350 million annually (King, 1994). The indiscriminate use of insecticides at all stages of the cotton crop has resulted in resurgence of pests especially of *H. armigera*. This pest has developed resistance to many groups of insecticides, particularly the synthetic pyrethroids (Castle et al., 1994; Armes et al., 1996). Also the adverse effect of insecticides on the natural enemy complex on cotton has compounded the bollworm management problem.

Indian regulators recently approved the commercial cultivation of bollworm-tolerant transgenic cotton, expressing an insecticidal protein (Cry1Ac) of *Bacillus thuringiensis (Bt)* (Jayaraman, 2002). The adoption of

*Corresponding author. Tel.: +91 80 3621189; fax: +91 80 3624343.
E-mail address: k.s.mohan@monsanto.com (K.S. Mohan).

0261-2194/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0261-2194(03)00170-4

Bt cotton by Indian farmers is likely to grow substantially and as a consequence the possibility of bollworms gaining resistance to the *in-planta* expressed Cry1Ac protein is an important concern. In contrast to traditional practices for chemical insecticides, insect resistance management (IRM) strategies are being proactively implemented in India from the earliest point of commercial introduction. Foremost among the IRM strategies is periodic monitoring of bollworm populations for changes in susceptibility to the Cry1Ac protein.

This study was conducted to establish a benchmark for the susceptibility of *H. armigera* populations from the major cotton growing regions in India in 1999 and 2001.

2. Materials and methods

2.1. Collection of H. armigera from field locations

Laboratory cultures were established by collecting 300 late instar larvae (III instar and above) of *H. armigera* from the cotton fields in the following locations (state in parenthesis) in the cotton belt of South and Central (1999 and 2001) and North (1999) India. (Fig. 1).

1999—Bathinda (Punjab), Sirsa (Harayana), Sri Ganganagar (Rajasthan), Khandwa (Madhya Pradesh), Anand (Gujarat), Akola (Maharashtra), Khammam and Guntur (Andhra Pradesh), Raichur, (Karnataka) and Coimbatore and (Tamil Nadu).

2001—Barwah and Khandwa (Madhya Pradesh), Rajkot, Vadodra (Gujarat), Jalgoan, Jalna, and Yavatmal (Maharashtra), Adilabad, Warangal, and Guntur (Andhra Pradesh), Davangere and Ranebennur (Karnataka) and Coimbatore and Dindigul (Tamil Nadu).

2.2. H armigera culture

The larvae were collected individually in glass vials containing semi-synthetic diet of Nagarkatti and Satyaprakash (1974) and transported to the laboratory of Project Directorate of Biological Control (ICAR), Bangalore. The larvae were transferred on to fresh diet upon arrival in the laboratory and allowed to pupate in the diet. The pupae were surface-sterilised in 0.1% sodium hypochlorite and kept aside for emergence of moths. About 250 moths representing each location were obtained. Each mating cage contained about 125 moths and eggs were collected as described by Kumar and Ballal (1990). The eggs were surface sterilised in 0.05% sodium hypochlorite solution and incubated for hatching. The F1 generation neonate larvae were used for bioassays.

2.3. Bioassay with standard Cry1Ac

The source of Cry1Ac standard was a freeze-dried commercial formulation of MVP® II (cell-cap® encapsulation system of Mycogen USA). The formulation contains 19.7% (by weight) of Cry1Ac protein. The *Bt* protein was assayed by the diet-incorporation method (Sims et al., 1996). Semi-synthetic diet for *H. armigera* diet was prepared (without formalin) in sterile glass bottles and the diet was kept warm in a hot water-bath at 60°C. The primary stock solution for Cry1Ac was prepared by thoroughly mixing (Vortex Cyclomixer) 5.076 mg MVP powder in 1 ml of 0.2% agar solution. Seven serial dilutions were prepared sequentially in 0.2% agar solution in sterile centrifuge tubes (40 ml) by diluting it to 1/4 of the previous concentration. The concentrations obtained were 8.0, 2.0, 0.5, 0.125, 0.031, 0.008 and 0.002 µg of Cry1Ac/ml of diet. A 5.2 ml diluted Cry1Ac standard, remaining in each of the serial dilution tubes, was thoroughly vortexed with 20.8 ml of warm diet (60°C) and approximately 1 ml was poured into each well of insect bioassay trays (CD International trays™, Massachusetts, USA). Newly hatched, active larvae were transferred onto the solidified diet in the bioassay trays with a fine hairbrush (1 larva/well). After larval transfer, bioassay trays were covered with self-adhesive pull-n-peel tabs (CD International pull-n-peel tabs™). The trays were kept in an incubator maintained at 27±0.5°C. Thirty-two larvae were used for each Cry1Ac concentration and untreated control. The entire assay was repeated 5 times with each population of *H. armigera*.

The bioassays were rated after seven days and observations on mortality, stadia of surviving larvae and group weight of all the surviving larvae were recorded. The larval stadia were determined on the basis of size of head capsules. Probit analysis of the data was carried out using JMP package version 3.1 (SAS Institute Inc., Cary, NC, USA) to compute lethal concentrations, LC_{50}, LC_{90} and moult inhibitory concentrations, MIC50, MIC90. Log linear regression analysis of the data was carried out to compute effective concentrations (weight stunting concentrations). From the regression curve 50% and 90% weight loss points were calculated and tabulated for EC_{50} and EC_{90}. Differences in response among populations were evaluated by non-overlapping of 95% fiducial limits for LC and MIC values.

3. Results

Populations of *H. armigera* collected in 1999 showed varied mortality response to Cry1Ac protein. The LC_{50} values for neonates ranged from 0.14 to 0.71 µg of Cry1Ac/ml of diet (Table 1). The population from

Bt cotton by Indian farmers is likely to grow substantially and as a consequence the possibility of bollworms gaining resistance to the *in-planta* expressed Cry1Ac protein is an important concern. In contrast to traditional practices for chemical insecticides, insect resistance management (IRM) strategies are being proactively implemented in India from the earliest point of commercial introduction. Foremost among the IRM strategies is periodic monitoring of bollworm populations for changes in susceptibility to the Cry1Ac protein.

This study was conducted to establish a benchmark for the susceptibility of *H. armigera* populations from the major cotton growing regions in India in 1999 and 2001.

2. Materials and methods

2.1. Collection of H. armigera from field locations

Laboratory cultures were established by collecting 300 late instar larvae (III instar and above) of *H. armigera* from the cotton fields in the following locations (state in parenthesis) in the cotton belt of South and Central (1999 and 2001) and North (1999) India. (Fig. 1).

1999—Bathinda (Punjab), Sirsa (Harayana), Sri Ganganagar (Rajasthan), Khandwa (Madhya Pradesh), Anand (Gujarat), Akola (Maharashtra), Khammam and Guntur (Andhra Pradesh), Raichur, (Karnataka) and Coimbatore and (Tamil Nadu).

2001—Barwah and Khandwa (Madhya Pradesh), Rajkot, Vadodra (Gujarat), Jalgoan, Jalna, and Yavatmal (Maharashtra), Adilabad, Warangal, and Guntur (Andhra Pradesh), Davangere and Ranebennur (Karnataka) and Coimbatore and Dindigul (Tamil Nadu).

2.2. H armigera culture

The larvae were collected individually in glass vials containing semi-synthetic diet of Nagarkatti and Satyaprakash (1974) and transported to the laboratory of Project Directorate of Biological Control (ICAR), Bangalore. The larvae were transferred on to fresh diet upon arrival in the laboratory and allowed to pupate in the diet. The pupae were surface-sterilised in 0.1% sodium hypochlorite and kept aside for emergence of moths. About 250 moths representing each location were obtained. Each mating cage contained about 125 moths and eggs were collected as described by Kumar and Ballal (1990). The eggs were surface sterilised in 0.05% sodium hypochlorite solution and incubated for hatching. The F1 generation neonate larvae were used for bioassays.

2.3. Bioassay with standard Cry1Ac

The source of Cry1Ac standard was a freeze-dried commercial formulation of MVP® II (cell-cap® encapsulation system of Mycogen USA). The formulation contains 19.7% (by weight) of Cry1Ac protein. The *Bt* protein was assayed by the diet-incorporation method (Sims et al., 1996). Semi-synthetic diet for *H. armigera* diet was prepared (without formalin) in sterile glass bottles and the diet was kept warm in a hot water-bath at 60°C. The primary stock solution for Cry1Ac was prepared by thoroughly mixing (Vortex Cyclomixer) 5.076 mg MVP powder in 1 ml of 0.2% agar solution. Seven serial dilutions were prepared sequentially in 0.2% agar solution in sterile centrifuge tubes (40 ml) by diluting it to 1/4 of the previous concentration. The concentrations obtained were 8.0, 2.0, 0.5, 0.125, 0.031, 0.008 and 0.002 µg of Cry1Ac/ml of diet. A 5.2 ml diluted Cry1Ac standard, remaining in each of the serial dilution tubes, was thoroughly vortexed with 20.8 ml of warm diet (60°C) and approximately 1 ml was poured into each well of insect bioassay trays (CD International trays™, Massachusetts, USA). Newly hatched, active larvae were transferred onto the solidified diet in the bioassay trays with a fine hairbrush (1 larva/well). After larval transfer, bioassay trays were covered with self-adhesive pull-n-peel tabs (CD International pull-n-peel tabs™). The trays were kept in an incubator maintained at 27±0.5°C. Thirty-two larvae were used for each Cry1Ac concentration and untreated control. The entire assay was repeated 5 times with each population of *H. armigera*.

The bioassays were rated after seven days and observations on mortality, stadia of surviving larvae and group weight of all the surviving larvae were recorded. The larval stadia were determined on the basis of size of head capsules. Probit analysis of the data was carried out using JMP package version 3.1 (SAS Institute Inc., Cary, NC, USA) to compute lethal concentrations, LC_{50}, LC_{90} and moult inhibitory concentrations, MIC50, MIC90. Log linear regression analysis of the data was carried out to compute effective concentrations (weight stunting concentrations). From the regression curve 50% and 90% weight loss points were calculated and tabulated for EC_{50} and EC_{90}. Differences in response among populations were evaluated by non-overlapping of 95% fiducial limits for LC and MIC values.

3. Results

Populations of *H. armigera* collected in 1999 showed varied mortality response to Cry1Ac protein. The LC_{50} values for neonates ranged from 0.14 to 0.71 µg of Cry1Ac/ml of diet (Table 1). The population from

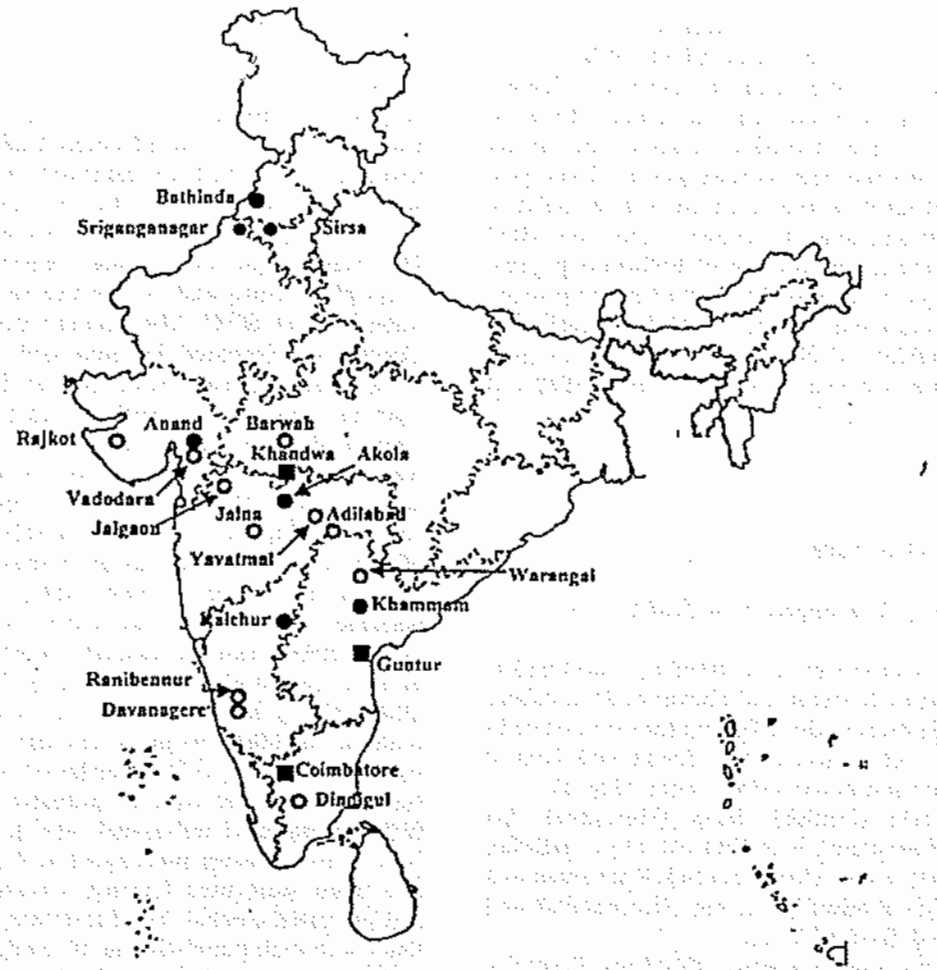


Fig. 1. Map of India showing locations from where *H. armigera* populations were collected in (a) 1999 (●); (b) 2001 (○); (c) both in 1999 & 2001

Table 1
Dose mortality response (LC) of *H. armigera* to Cry1Ac—1999 populations

Location	State	LC_{50}	95% fiducial limit		LC_{90}	95% fiducial limit		Slope±SE	χ^2
			Lower	Upper		Lower	Upper		
Bathinda	Punjab	0.14^{d}	0.09	0.21	1.27^{c}	0.70	3.21	2.41±0.31	61.03
Sirsa	Haryana	0.49^{ab}	0.32	0.78	5.57^{ab}	2.83	16.22	2.11±0.27	59.78
Sriganganagar	Rajasthan	0.61^{ab}	0.41	0.93	4.52^{ab}	2.55	11.10	2.58±0.35	55.08
Khandwa	Madhya Pradesh	0.32^{bc}	0.21	0.48	2.32^{bc}	1.33	5.43	2.57±0.34	58.67
Anand	Gujarat	0.58^{ab}	0.38	0.93	5.70^{ab}	2.84	15.91	2.16±0.28	59.12
Akola	Maharashtra	0.28^{c}	0.18	0.43	2.87^{ab}	1.51	7.76	2.20±0.28	61.11
Khammam	Andhra Pradesh	0.71^{a}	0.46	1.11	6.94^{a}	3.59	19.96	2.32±0.32	55.61
Guntur	Andhra Pradesh	0.30^{bc}	0.19	0.50	5.25^{ab}	2.42	18.11	2.07±0.27	60.71
Raichur	Karnataka	0.71^{a}	0.48	1.06	4.71^{ab}	2.70	11.41	2.76± 0.39	52.45
Coimbatore	Tamil Nadu	0.19^{cd}	0.13	0.27	1.17^{c}	0.69	2.60	3.01±0.42	55.61

χ^2 significant at $p=0.0001$ level of significance. LC_{50} and LC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits; LC_{50}—concentration of Cry1Ac (μg/ml) needed to kill 50% of test larval population in the observation period of seven days. Similarly LC_{90} is the concentration of Cry1Ac which would be required to kill 90% of test population.

Bathinda (Punjab) had the lowest LC_{50} value, whereas populations from Khammam and Raichur, both from the state of Andhra Pradesh, had the highest LC_{50} values. Populations have been compared based on non-overlap of 95% fiducial limits in Table 1. The LC_{90} values ranged from 1.17 to 6.94 μg of Cry1Ac/ml of diet.

Table 2
Dose mortality response (LC) of *H. armigera* to Cry1Ac—2001 populations

Location	State	LC_{50}	95% fiducial limit		LC_{90}	95% fiducial limit		Slope ± SE	χ^2
			Lower	Upper		Lower	Upper		
Barwah	Madhya Pradesh	0.19^{a}	0.12	0.28	1.47^{bc}	0.83	3.53	2.52 ± 0.328	59.68
Khandwa	Madhya Pradesh	0.26^{a}	0.17	0.39	2.07^{bc}	1.15	4.93	2.46 ± 0.322	60.17
Rajkot	Gujarat	0.61^{a}	0.04	0.95	5.58^{ab}	2.96	15.43	1.76 ± 0.306	56.84
Vadodra	Gujarat	0.20^{a}	0.13	0.30	1.43^{bc}	0.83	3.30	2.64 ± 0.346	58.74
Jalgoan	Maharashtra	0.40^{a}	0.26	0.65	6.70^{a}	3.04	24.21	1.96 ± 0.258	59.16
Jalna	Maharashtra	0.31^{a}	0.21	0.46	1.92^{bc}	1.14	4.26	2.75 ± 0.366	56.93
Yavatmal	Maharashtra	0.22^{a}	0.15	0.32	1.57^{bc}	0.90	3.69	2.77 ± 0.376	56.96
Adilabad	Andhra Pradesh	0.15^{a}	0.09	0.23	1.47^{bc}	0.80	3.67	2.30 ± 0.290	63.31
Warangal	Andhra Pradesh	0.20^{a}	0.13	0.31	1.95^{bc}	1.06	5.01	2.55 ± 0.344	59.38
Guntur	Andhra Pradesh	0.21^{a}	0.14	0.31	1.44^{bc}	0.84	3.29	2.27 ± 0.358	58.28
Ranebennur	Karnataka	0.14^{a}	0.10	0.21	1.02^{c}	0.59	2.29	2.58 ± 0.334	59.99
Davanagere	Karnataka	0.11^{a}	0.07	0.17	1.09^{c}	0.57	3.07	3.13 ± 0.460	54.27
Coimbatore	Tamil Nadu	0.16^{a}	0.11	0.25	1.17^{c}	0.69	2.65	2.57 ± 0.330	60.75
Dindigul	Tamil Nadu	0.19^{a}	0.12	0.31	2.61^{ab}	1.33	7.32	1.97 ± 0.240	64.43

χ^2 significant at $p = 0.0001$ level of significance. LC_{50} and LC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits; LC_{50}—concentration of Cry1Ac (μg/ml) needed to kill 50% of test larval population in the observation period of seven days. Similarly LC_{90} is the concentration of Cry1Ac which would be required to kill 90% of test population.

Table 3
Moult inhibitory concentration response of *H. armigera* to Cry1Ac—1999 populations

Location	State	MIC_{50}	95% fiducial limit		MIC_{90}	95% fiducial limit		Slope ± SE	χ^2
			Lower	Upper		Lower	Upper		
Bathinda	Punjab	0.05^{d}	0.03	0.07	0.33^{c}	0.19	0.75	2.72 ± 0.37	55.42
Sirsa	Haryana	0.18^{ab}	0.12	0.28	1.46^{ab}	0.83	3.46	2.47 ± 0.32	59.43
Sriganganagar	Rajasthan	0.20^{ab}	0.14	0.29	1.13^{ab}	0.69	2.42	2.93 ± 0.39	55.75
Khandwa	Madhya Pradesh	0.15^{bc}	0.10	0.22	0.77^{ab}	0.48	1.60	3.14 ± 0.43	53.23
Anand	Gujarat	0.19^{ab}	0.13	0.29	1.58^{a}	0.87	4.00	2.37 ± 0.31	59.55
Akola	Maharashtra	0.12^{c}	0.08	0.18	1.02^{ab}	0.57	2.49	2.30 ± 0.30	60.74
Khammam	Andhra Pradesh	0.23^{ab}	0.16	0.34	1.25^{ab}	0.77	2.68	3.07 ± 0.42	54.06
Guntur	Andhra Pradesh	0.12^{c}	0.08	0.19	1.31^{ab}	0.70	3.34	2.35 ± 0.27	61.51
Raichur	Karnataka	0.27^{a}	0.19	0.38	1.26^{ab}	0.80	2.61	3.23 ± 0.45	51.50
Coimbatore	Tamil Nadu	0.10^{cd}	0.06	0.08	0.51^{bc}	0.32	1.07	3.04 ± 0.41	54.50

χ^2 significant at $p = 0.0001$ level of significance. MIC_{50} and MIC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits; MIC_{50}—concentration of Cry1Ac (μg/ml) that will inhibit moulting of I-instar larvae into II instar, of 50% of test larval population in the observation period of seven days. In other words the affected larvae are so severely retarded that they stay as I instar during assay period of 7 days. Similarly MIC_{90} is the concentration that will inhibit moulting of 90% of test population.

Similarly, Table 2 gives LC_{50} and LC_{90} values of *H. armigera* populations from 14 locations in 2001. The LC_{50} and LC_{90} values in 2001 were relatively lower than those obtained in 1999. The LC_{50} values ranged from 0.11 of Davanagere population (Karnataka) to 0.61 from Rajkot (Gujarat). Study of overlap of fiducial limits (at $P = 0.95$) of LC_{50} values indicated that there was some variability among different populations of 1999 and no variability among the 2001 populations.

The moult inhibitory concentration (MIC) is that dose of *Bt* protein, which will inhibit progress of neonates from I into the II instar. The affected larvae remain in the first instar even on the eighth day of observation. The MIC_{50} and MIC_{90} values of the populations of 1999 and 2001 are given in Tables 3 and 4, respectively. MIC_{50} values of the 1999 collection ranged from 0.05 to 0.27 μg of Cry1Ac/ml of diet and those of 2001 collection ranged from 0.05 to 0.14 and fiducial limits (at $P = 0.95$) of the data indicated that there was significant variability in response among different populations. EC_{50} values ranged from 0.003 to 0.008 and from 0.0003 to 0.004 for 1999 and 2001 populations (Tables 5 and 6), respectively.

4. Discussion

B. thuringiensis and its multitude of insecticidal proteins are among the important alternatives to chemical insecticides. The evolution of resistance in the

Table 4
Moult inhibitory concentration (MIC) response of *H. armigera* to Cry 1 Ac—2001 populations

Location	State	MIC_{50}	95% fiducial limit		MIC_{90}	95% fiducial limit		Slope±SE	χ^2
			Lower	Upper		Lower	Upper		
Barwah	Madhya Pradesh	0.09^{ab}	0.06	0.14	0.62^{ab}	0.36	1.39	2.81±0.376	56.35
Khandwa	Madhya Pradesh	0.10^{ab}	0.07	0.15	0.65^{ab}	0.39	1.42	2.90±0.392	55.94
Rajkot	Gujarat	0.13^{ab}	0.09	0.19	0.69^{ab}	0.43	1.45	2.90±0.388	56.0
Vadodra	Gujarat	0.05^{cd}	0.04	0.08	0.28^{bc}	0.17	0.59	3.11±0.434	52.09
Jalgoan	Maharashtra	0.13^{ab}	0.09	0.19	0.91^{a}	0.53	2.08	2.69±0.36	58.94
Jalna	Maharashtra	0.14^{a}	0.10	0.20	0.55^{ab}	0.37	1.11	3.72±0.548	46.59
Yavatmal	Maharashtra	0.08^{bc}	0.05	0.12	0.46^{ab}	0.28	0.98	2.88±0.394	53.80
Adilabad	Andhra Pradesh	0.07^{bc}	0.05	0.11	0.41^{ab}	0.25	0.88	2.92±0.392	55.92
Warangal	Andhra Pradesh	0.09^{ab}	0.06	0.15	0.48^{ab}	0.30	1.00	3.31±0.468	51.82
Guntur	Andhra Pradesh	0.12^{ab}	0.09	0.17	0.53^{ab}	0.34	1.05	3.54±0.514	48.69
Ranebennur	Karnataka	0.07^{c}	0.05	0.10	0.36^{ab}	0.22	0.74	3.17±0.440	52.47
Davanagere	Karnataka	0.05^{cd}	0.03	0.07	0.26^{bc}	0.16	0.54	3.58±0.542	49.51
Coimbatore	Tamil Nadu	0.05^{cd}	0.03	0.08	0.25^{c}	0.15	0.51	3.40±0.490	51.20
Dindigul	Tamil Nadu	0.07^{bc}	0.05	0.11	0.64^{ab}	0.36	1.53	2.28±0.290	61.86

χ^2 significant at $p=0.0001$ level of significance. MIC_{50} and MIC_{90} values designated by different letters are significantly different from each other through non-overlap of 95% fiducial limits; MIC_{50}—concentration of Cry1Ac (μg/ml) that will inhibit moulting of I instar larvae into II instar, of 50% of test larval population in the observation period of seven days. In other words the affected larvae are so severely retarded that they stay as I instar during assay period of 7 days. Similarly MIC_{90} is the concentration that will inhibit moulting of 90% of test population.

Table 5
Weight stunting concentration response of *H. armigera* to Cry1Ac—1999 populations

Location	State	EC_{50}	Lower limit	Upper limit	EC_{90}	Lower limit	Upper limit
Bathinda	Punjab	0.004	0.003	0.005	0.031	0.014	0.167
Sirsa	Haryana	0.004	0.002	0.005	0.068	0.035	0.197
Sriganganagar	Rajasthan	0.003	0.003	0.003	0.039	0.025	0.072
Khandwa	Madhya Pradesh	0.005	0.004	0.006	0.053	0.044	0.074
Anand	Gujarat	0.004	0.003	0.005	0.062	0.041	0.100
Akola	Maharashtra	0.004	0.003	0.004	0.029	0.019	0.052
Khammam	Andhra Pradesh	0.004	0.003	0.005	0.051	0.030	0.102
Guntur	Andhra Pradesh	0.008	0.006	0.010	0.076	0.045	0.146
Raichur	Karnataka	0.003	0.002	0.003	0.038	0.027	0.057
Coimbatore	Tamil Nadu	0.003	0.003	0.004	0.042	0.032	0.065

EC—Effective concentration (related to stunting—weight related). EC_{50}, EC_{90}—Concentration of Cry1Ac (μg/ml of diet) that would stunt the larvae such that they weighed 50 and 10% of that of larvae in the untreated control group.

target pests to these proteins is an important issue. So far field resistance has been observed in diamondback moth for a *Bt* spray formulation (Liu et al., 1995; Shelton et al., 1993), but laboratory selection experiments have shown the potential of many lepidopteran pests to develop resistance to *Bt* (Ferre and Van Rie, 2002) insecticidal proteins.

Variation in LC_{50} and MIC_{50} values within the populations screened during 1999 or 2001 is limited to a five-fold change. Since the locations of 1999 and 2001 were not same (except Khandwa, Guntur and Coimbatore), a meaningful comparison across the years for the same location cannot be made. There were no obvious 'location specific' values. Also there was no significant differences in the susceptibility values between 1999 and 2001 collections.

Similar baseline susceptibility studies carried-out in the USA showed that variation in LC_{50} among 12 field populations of *H. virescens* and 15 populations of *H. zea* was 16-fold for Cry1Ac and 13-fold for 'Dipel' (Stone and Sims, 1993). In another study, variation among 16 colonies of *H. virescens* with 'Dipel' was 71-fold and among 11 colonies of *H. zea* with Cry1Ac was 441-fold (Luttrell et al., 1999). Studies conducted in China have revealed variation in LC_{50} to an extent of 100-fold with Cry1Ac in 23 populations of *H. armigera* (Wu et al., 1999) and 4-fold with the HD-1 strain of *Bt* var. *kurstaki*, among 8 populations of the same insect pest (Zhao et al., 1996). The variation in LC_{50} values among Indian populations of *H. armigera*, seen in the current study, is small (about five-fold) when compared to the variability reported from other countries. Gujar et al (2000) studied susceptibility of Indian populations of *H. armigera* to the HD-1 strain of *Bt* var. *kurstaki* and reported variations in susceptibility among populations collected from various locations and from various crops

Table 6
Weight stunting concentration response of *H. armigera* to Cry1Ac—2001 populations

Location	State	EC_{50}	Lower limit	Upper limit	EC_{90}	Lower limit	Upper limit
Barwah	Madhya Pradesh	0.002	0.002	0.002	0.016	0.014	0.019
Khandwa	Madhya Pradesh	0.003	0.003	0.005	0.047	0.020	0.262
Rajkot	Gujarat	0.001	0.000	0.002	0.028	0.014	0.090
Vadodra	Gujarat	0.002	0.002	0.002	0.014	0.011	0.018
Jalgoan	Maharashtra	0.004	0.003	0.005	0.028	0.019	0.044
Jalna	Maharashtra	0.002	0.001	0.002	0.030	0.020	0.050
Yavatmal	Maharashtra	0.0003	0.0002	0.0004	0.010	0.008	0.016
Adilabad	Andhra Pradesh	0.001	0.000	0.001	0.019	0.013	0.031
Warangal	Andhra Pradesh	0.002	0.001	0.002	0.032	0.023	0.045
Guntur	Andhra Pradesh	0.003	0.002	0.004	0.054	0.026	0.054
Ranebennur	Karnataka	0.003	0.002	0.004	0.043	0.027	0.111
Davanagere	Karnataka	0.0004	0.0002	0.0005	0.013	0.010	0.021
Coimbatore	Tamil Nadu	0.001	0.000	0.001	0.009	0.009	0.010
Dindigul	Tamil Nadu	0.001	0.001	0.001	0.014	0.012	0.018

EC—Effective concentration (related to stunting—weight related). EC_{50}, EC_{90}—Concentration of Cry1Ac (μg/ml) that would stunt the larvae such that they weighed 50 and 10% of that of larvae in the untreated control group.

such as pigeonpea, cotton, okra and tomato. In the reported study, susceptibility values are reported in terms of % mortality in response to two fixed doses of 10 and 100 ppm of *Btk* HD-1 (% mortality ranged from 8.72% to 80.8% and 38.9% to 93.7%, respectively) and hence comparisons cannot be made with the current study. Our study reports detailed baseline-susceptibility analyses of *H. armigera* populations from the cotton growing areas in India, for two years, prior to the commercial launch of *Bt* cotton in India.

Bt cotton was given regulatory approval in March 2002 and the crop was commercially cultivated from the kharif (growing) season of 2002. It is now possible to monitor development of resistance to Cry1Ac with reference to the baseline values generated in this study.

Acknowledgements

The authors are thankful to Maharashtra Hybrid Seeds Company Ltd., Mumbai for financial assistance for conducting the study; Sakuntala Sivasupramaniam and John Greenplate of Monsanto Company, USA, and the members of Crop Protection Division, Monsanto Research Center, Bangalore for technical inputs.

References

Armes, N.J., Jadhav, D.R., De Souza, K.R., 1996. A survey of insecticide resistance in *Helicoverpa armigera* in the Indian subcontinent. Bull. Entomol. Res. 86, 499–514.

Castle, S.J., Prabhakar, N., Henneberry, T., 1994. Insecticide resistance and its management in cotton insects. ICAC review article on cotton production research No.5, ICAC, Washington, USA.

Dhawan, A.K., Sidhu, A.S., Simwat, G.S., 1988. Assessment of avoidable loss in cotton (*Gossypium arboreum*) due to sucking pests and bollworms. Indian J. Agric. Sci. 58, 290–292.

Ferre, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47, 501–533.

Gujar, G.T., Kumari, A., Kalia, V., Chandrashekar, K., 2000. Spatial and temporal variation in susceptibility of the American bollworm, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* var. *kurstaki* in India. Curr. Sci. 78, 995–1004.

Jayaraman, 2002. India approves GM cotton. Nature Biotech 20 (5), 415.

King, A.B.S., 1994. In: Matthews, G.A., Tunstall, J.P. (Eds.), Insect Pests of Cotton. Commonwealth Agricultural Bureau International, UK, pp. 39–106.

Kumar, P., Ballal, C.R., 1990. An improved ovipositional cage and egg collection technique for mass multiplication of *Helicoverpa armigera* (Hubner). Indian J. Plant Prot. 18, 255–259.

Liu, Y.B., Tabashnik, B.E., Johnson, M.W., 1995. Larval age affects resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econom. Entomol. 88, 788–792.

Luttrell, R.G., Wan, L., Knighten, K., 1999. Variation in susceptibility of Noctuid (Lepidoptera) larvae attacking cotton and soybean to purified endotoxin proteins and commercial formulations of *Bacillus thuringiensis*. J. Econom. Entomol. 92, 21–32.

Nagarkatti, S., Satyaprakash., 1974. Rearing of *Heliothis armigera* (Hb.) on artificial diet. Tech. Bull. Commonwealth Inst. Biol. Control 17, 169–173.

Satpute, U.S., Sarnaik, D.N., Bhakrao, P.D., 1988. Assessment of avoidable field losses in cotton yield due to sucking pests and bollworms. Indian J. Plant Prot. 16, 37–39.

Shelton Jr., A.M., Robertson, J.L., Tang, J.D., Perez, C., Eigenbrode, S.D., Preisler, H.K., Wilsey, W.T., Cooley, R.J., 1993. Resistance of diamondback moth (Lepidoptera: Plutellidae) to *Bacillus thuringiensis* subspecies in the field. J. Econom. Entomol. 86, 697–705.

Sims, S.R., Greenplate, J.T., Stone, T.B., Caprio, M.A., Gould, F.L., 1996. Monitoring strategies for early detection of Lepidoptera resistance to *Bacillus thuringiensis* insecticidal proteins. In: Brown, T.M. (Ed.), Molecular Genetics and Evolution of Pesticide Resistance, An American Chemical Society Publication, Washington, USA. American Chemical Society Symposium Series No. 645, pp. 230–242.

Stone, T.B., Sims, S.R., 1993. Geographic susceptibility of *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis*. J. Econom. Entomol. 86, 989–994.

Sundaramurthy, V.T., 1986. Insect management in cotton system. In: Jayaraj, S. (Ed.), Pest and Disease Management : Oilseeds, Pulses, Millets and Cotton, Tamil Nadu Agricultural University, Coimbatore, pp. 189–194.

Wu, K., Guo, Y., Lv, N., 1999. Geographical variations of susceptibility of *Helicoverpa armigera* (Lepidoptera: Noctuidae) to *Bacillus thuringiensis* insecticidal protein in China. J. Econom. Entomol. 92, 273–278.

Zhao, J.Z., Lu, M.G., Fan, X.L., Wei, C., Liang, G.M., Zhu, C.C., 1996. Resistance monitoring of *Helicoverpa armigera* to *Bacillus thuringiensis* in North China. Resist. Pest. Manage. Newsl. 8, 46–47.

Relative Abundance of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on Different Host Crops in India and the Role of These Crops as Natural Refuge for *Bacillus thuringiensis* Cotton

K. C. RAVI,[1] K. S. MOHAN,[1] T. M. MANJUNATH,[1] C. HEAD,[2] B. V. PATIL,[3] D. P. ANGELINE CREBA,[4] K. PREMALATHA,[4] J. PETER,[5] AND N.G.V. RAO[6]

Monsanto Research Center, Bangalore, Karnataka 560092, India

Environ. Entomol. 34(1): 59–69 (2005)

ABSTRACT *Helicoverpa armigera* (Hübner) infests many economically important crops in India, including cotton, pigeonpea, chickpea, sunflower, corn, chili, tomato, and okra. These crops are cultivated in proximity to each other in central and southern India. The current study examined the relative abundance of *H. armigera* on different host crops within a crop mosaic. Field studies conducted over two growing seasons (2000–2001 and 2001–2002) indicated differences in egg and larval densities among the host plant species. All of the host crops supported eggs and larvae of *H. armigera*, but the populations on pigeonpea and chickpea were significantly greater than on cotton and other host crops. Egg numbers also were significantly higher on sunflower, okra, and tomato than on cotton, but larval numbers were not significantly different from cotton at comparable times. Both egg and larval numbers on corn and chili were not significantly different from those on cotton. This study demonstrates that a number of host crops of *H. armigera* support large populations at the same time that cotton is infested. Thus, these crops may act as important sources of refuge for *Bacillus thuringiensis* cotton plantings in central and southern India.

KEY WORDS cotton bollworm, alternative hosts, smallholders, insect resistance management

The noctuid *Helicoverpa armigera* (Hübner) is a major pest of many economically important crops in India, including cotton, pigeonpea, chickpea, sunflower, tomato, sorghum, millet, okra, and corn (Manjunath et al. 1989, Sharma 2001). In particular, *H. armigera* is the predominant bollworm on Indian cotton, causing 14–56% damage (Kaushik et al. 1969, Manjunath et al. 1989, Jairaj 1990). Fifty-four percent of the total insecticides used on all crops in India are used on cotton, and most of these are directed against *H. armigera* (Mohan and Manjunath 2002). As a consequence, this pest has evolved resistance to many insecticides in India (Armes et al. 1996, Kranthi 1997).

A novel tool is now available to control *H. armigera* in the form of cotton genetically engineered to express an insecticidal protein, Cry1Ac, derived from the bacterium *Bacillus thuringiensis* (Berliner) (Bt). These transgenic varieties (Bt cotton) provide effective control of *H. armigera* and other bollworms such as *Earias vittella* (F.) and pink bollworm, *Pectinophora gossypiella* (Saunders). Bt cotton varieties have now been registered for commercial use in the United States, Australia, Mexico, Colombia, Argentina, China, India, and South Africa. A critical part of the introduction of Bt cotton is to ensure that it is used appropriately and judiciously. One element of this product stewardship is the implementation of management strategies to slow the rate at which target insect species such as *H. armigera* evolve resistance to the Cry1Ac protein. The resistance management strategy for Bt cotton critically depends upon the provision of refuges of non-Bt plants where populations of susceptible target insects may build to mate with any rare resistant insects that emerge from Bt cotton. In countries where cotton is grown intensively on large, relatively homogeneous farms (such as in the United States), farmers planting Bt cotton also are required to plant refuges of conventional cotton. However, where farm sizes are smaller and cropping systems are more diverse, as in much of Asia and Africa, several other crop (other than cotton) species that can support the target pests of Bt cotton may be an important source of refuge inherent to these systems. If the target pests are using a variety of these alternative host plant species, and they are not being controlled using Bt on these other

[1] Monsanto Research Center, #41/2A, Vasanth's Business Park, Bellary Rd., NH-7, Hebbal, Bangalore 560092, India.
[2] Monsanto LLC, A2NA, 800 N. Lindbergh Blvd., St. Louis, MO 63167.
[3] College of Agriculture, University of Agricultural Sciences (Dharwad), Raichur 584101, India.
[4] Tamil Nadu Agricultural University, Coimbatore, India.
[5] Nagarjuna Agricultural Research and Development Institute, Hyderabad 500082, India.
[6] Punjab Rao Deshmukh Krishi Vidyapeeth, Krishinagar, Akola 444104, India.

0046-225X/05/0059–0069$04.00/0 © 2005 Entomological Society of America

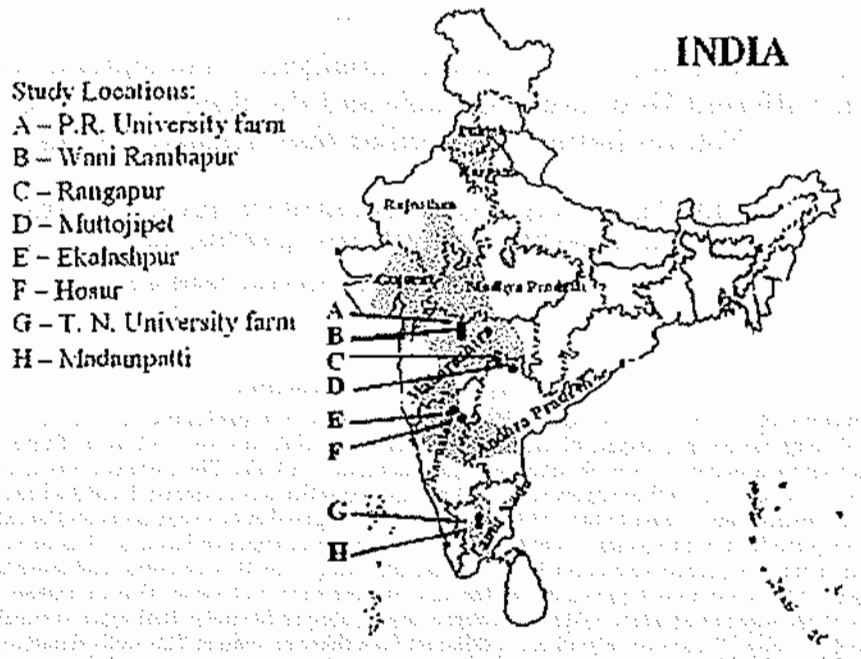


Fig. 1. Study locations in major cotton-growing areas (shaded area) in central and southern India.

hosts, then structured refuges for Bt crops may not be necessary under these conditions. In these cases, both cropping practices and the degree of polyphagy of the target insect species are important.

The case of *H. armigera* in India seems to be particularly amenable to resistance management through an approach based around alternative hosts. This insect can be found on ≈180 plant species other than cotton, including many of the major pulse crops, many vegetables, and both dicotyledonous and monocotyledonous species (Manjunath et al. 1989). In particular, pulse crops such as chickpea and pigeonpea are major hosts of *H. armigera* and are planted on larger areas than cotton (Directorate of Economics and Statistics 2003). Furthermore, the Indian agricultural landscape is highly fragmented, and many alternative crop hosts of *H. armigera* are cultivated alongside cotton. The crop phenology in India ensures the presence of five to six alternative hosts of the pest in any given part of the growing season (Manjunath et al. 1989, Khadi et al. 2003). However, for this approach to be viable, a number of conditions must hold:

1. The target pest species must use multiple host plant species that overlap in both space and time. This has been found to occur for *H. armigera* under natural conditions (Manjunath et al. 1989).
2. The attractiveness of the different host plant species and the pest population dynamics on these hosts must be comparable to allow the different alternative hosts to produce sufficient susceptible insects at the right time to interbreed with any resistant insects emerging from the Bt cotton.
3. The distribution of these different host plant species must overlap at a sufficiently fine scale and consistently enough to act as a functional refuge in all relevant cotton-growing regions.
4. The pest insects must move between the different host plant species and individuals produced on one host must be capable of mating with individuals produced on other hosts.

In this study, we examined whether the first two of these conditions hold for *H. armigera* in the cotton belts of central and southern India. We quantified the population sizes of *H. armigera* on adjoining fields of different host crops, including cotton, pigeonpea, chickpea, corn, sunflower, tomato, and okra, at locations throughout the cotton belts of central and south India over the course of 2 yr. In a separate study, we have used satellite mapping to examine the third of the conditions (K.C.R. et al., unpublished data).

Materials and Methods

Study Locations. Selected areas had to contain cotton fields, along with any two alternative hosts of *H. armigera* in adjoining fields. Each crop occupied an area of at least 0.4 ha (1 acre) at each location. All the locations were within intensive cotton-growing areas where the pest incidence typically is high during the growing season. The general cropping pattern was the same each year in a given location. Six of the eight locations were in farmers' fields, whereas the other two locations belonged to universities. Agronomic practices were as per local farmer practice.

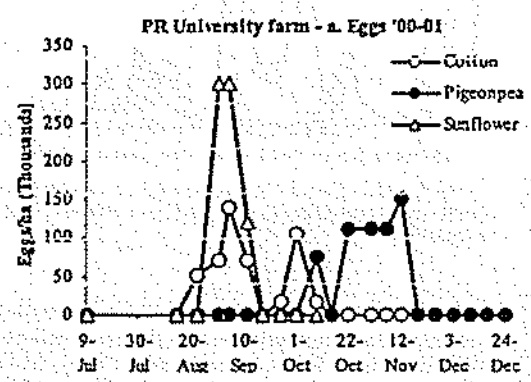
Table 1. Details of the locations and crops selected for the study

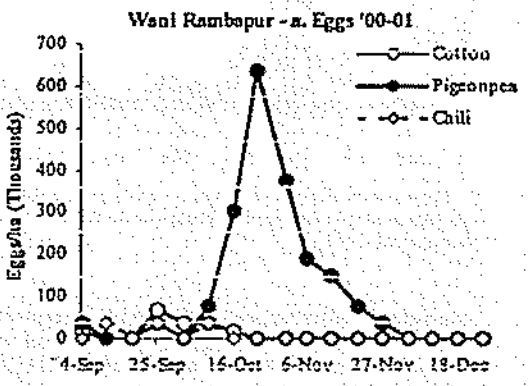
Collaborating institution	Location	Crop	
		2000–2001	2001–2002
Punjabrao Deshmukh Krishi Vignan Peeth, Akola, Maharashtra State	PR University farm	Cotton, pigeonpea, sunflower	
	Wani Rambapur	Cotton, pigeonpea, chili	
Nagarjuna Agriculture Research and Development Institute, Hyderabad, Andhra Pradesh State	Rangapur		Cotton, chili, corn
	Muttojipet		Cotton, chili, corn
College of Agriculture, University of Agricultural Sciences, Dharwad, Karnataka State	Ekalashpur	Cotton, pigeonpea, chickpea, sunflower, sorghum	Cotton, pigeonpea, chickpea, sunflower, sorghum
	Hosur	Cotton, pigeonpea, sunflower, sorghum	Cotton, pigeonpea, chickpea, sorghum
Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu State	TN University farm Madampatti	Cotton, pigeonpea, chickpea Cotton, okra, tomato	Cotton, pigeonpea, chickpea Cotton, okra, tomato

The study was carried out during the main cotton-growing season (Kharif) in both years. In general, all of the crops at a given location are planted at a similar time, taking advantage of prevailing soil moisture; farmers, in general, prepare the land with the first showers followed by sowing with second rains. Even in areas under irrigation, crops are sown at a similar time to use the rainwater. Specifically, pigeonpea, okra, and tomato are typically planted with cotton. Most of the study locations are rain-fed, and the only crops grown under ensured irrigation are rice, wheat, and certain vegetables, none of which are focal crops for this study.

The following is a brief description of the nature and planting details at the individual locations, moving from the most northern location to the most southern location (Fig. 1; Table 1)

Punjabrao Deshmukh Krishi Vignan Peeth. This university farm is located within a region where cotton





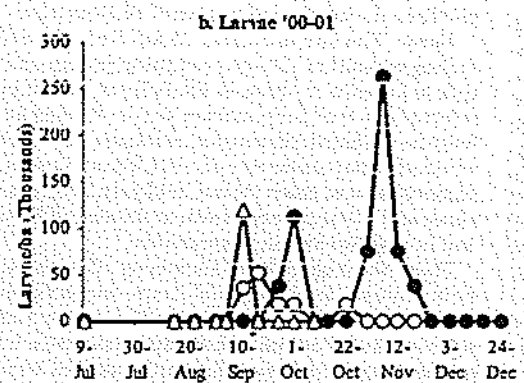


Fig. 2. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) on the Punjab Rao University farm in Maharashtra during 2000–2001.

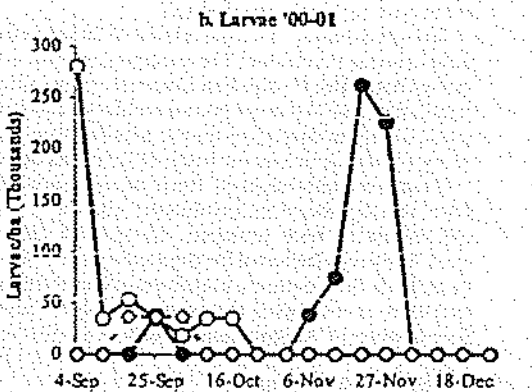
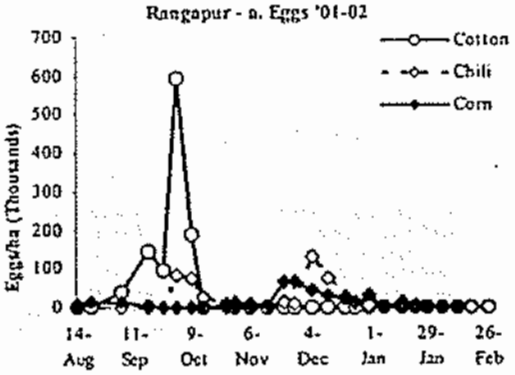
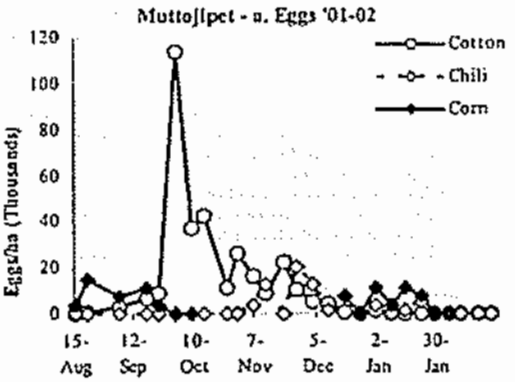


Fig. 3. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) at Wani Rambapur in Maharashtra during 2000–2001.





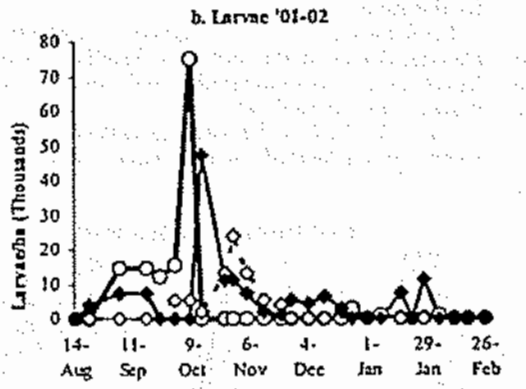


Fig. 4. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) at Rangapur in Andhra Pradesh during 2001–2002.

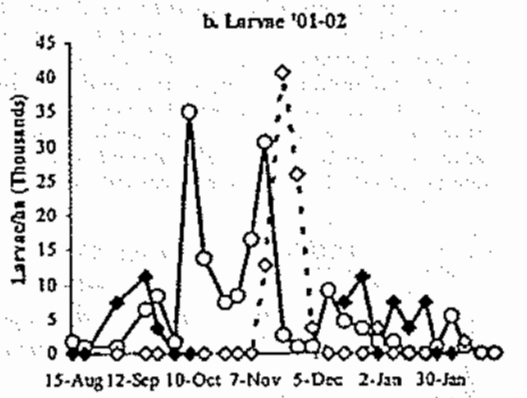


Fig. 5. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) at Muttojipet in Andhra Pradesh during 2001–2002.

commonly is intercropped with pigeonpea and also is cultivated as a sole crop. Pigeonpea, sunflower, chili, and corn also are cultivated as sole crops. The crops selected for the study (cotton, sunflower, and pigeonpea) were sown during the second week of July and cultivated on black, rain-fed soil.

Wani Rambapur. This site is located 30 km from the collaborating institution. The cropping pattern is similar to that at the Punjab Rao University farm. Cotton, pigeonpea, and chili were grown on black, rain-fed soil and were sown during the fourth week of May, fourth week of June, and second week of July, respectively.

Rangapur and Muttojipet. Both locations are 160 km from the collaborating institution. Cotton is grown extensively in both locations, along with chili and corn. The crops included for the study were cultivated on red soil. Both cotton and chili were under irrigation and were sown during the second week of July and fourth week of August, respectively. Two successive crops of corn were sown during the first week of July and first week of October.

Ekalashpur. This site is located 5 km from the collaborating institution. This region is characterized by cotton intercropped with pigeonpea, as well as sole crops of pigeonpea, sunflower, chickpea, sorghum, and corn. The crops selected for the study were cultivated on deep black, rain-fed soil. Cotton and pigeonpea were sown during the second week of July, sorghum during the fourth week of October, and chickpea during the second week of November.

Hosur. This site is located 15 km from the collaborating institution. General cropping pattern and soil type are similar to those at Ekalashpur. Of the crops selected for the study, cotton was cultivated under irrigation, whereas pigeonpea, chickpea, and sorghum were cultivated under rain-fed conditions. Cotton and pigeonpea were sown during mid-July, whereas sorghum and chickpea were sown at the end of October and in mid-November, respectively.

Tamil Nadu Agricultural University. This university farm contains many types of crops, including many vegetables, cotton, and cereals. The selected crops were cultivated on red soil. Cotton was grown under irrigation and was sown during the second week of August, whereas pigeonpea and chickpea were rain-fed and were sown during the fourth week of June and second week of November, respectively.

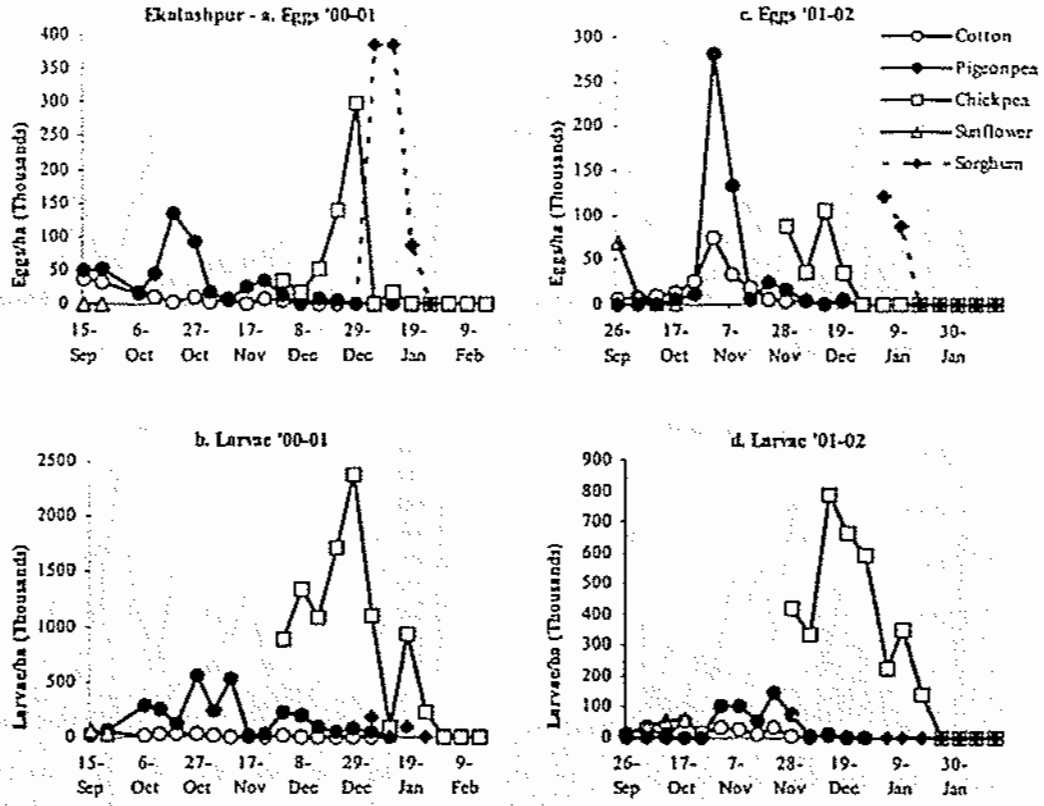


Fig. 6. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) during 2000–2001 and (c) eggs and (d) larvae during 2001–2002 at Ekalashpur in Karnataka.

Madampatti. This site is located in a red soil area, 25 km from the collaborating institution. Cotton is one of the predominant crops, along with vegetables and corn. The crops chosen for the study were grown under irrigated condition. Cotton was sown during the last week of August. Two successive crops of tomato and okra were included. The first crop of tomato was sown during the first week of September, whereas the second crop was sown in the last week of November. The two crops of okra were sown during the last week of August and the last week of November, respectively.

Sampling. Counting of immature stages was restricted to eggs and larvae of *H. armigera.* Pupal counts could not be carried out because of practical problems associated with recovery of pupae from the soil. Counts were carried out every 15 d, beginning 30 d after sowing and lasting until harvest. During each count, eggs present up to 10 cm from the tip of the plant and the number of larvae on the entire plant were recorded for 20 randomly selected plants in each field. Plants sampled once were tagged and not sampled again.

Analysis. Counts of *H. armigera* at each sampling time were converted to numbers per hectare by using the plant density of each crop. Eggs and larvae also were observed on the weed *Lagascea mollis* Cavanilles (Compositae), found growing around the experimental plots. However, the populations on this weed could not be quantified on a per hectare basis because of difficulty in estimating the weed population size.

The numbers of eggs and larvae on cotton were compared with the numbers on other crop hosts in a particular location at the same time. For a given pair of crops, this analysis was performed across all locations where both crops were present. Wilcoxon's signed rank test was used because of the irregular data distributions (Sigmastat 2.0, Jandel Corporation 1995). Only sampling times when insects were present on one or both of the crops were included. Sorghum was not analyzed because insect populations were only present for a short time on this crop.

Results

H. armigera egg and larval populations on different host crops over the course of the growing season are presented for the eight sites in Figs. 2–9. The mean number of *H. armigera* eggs and larvae recorded on

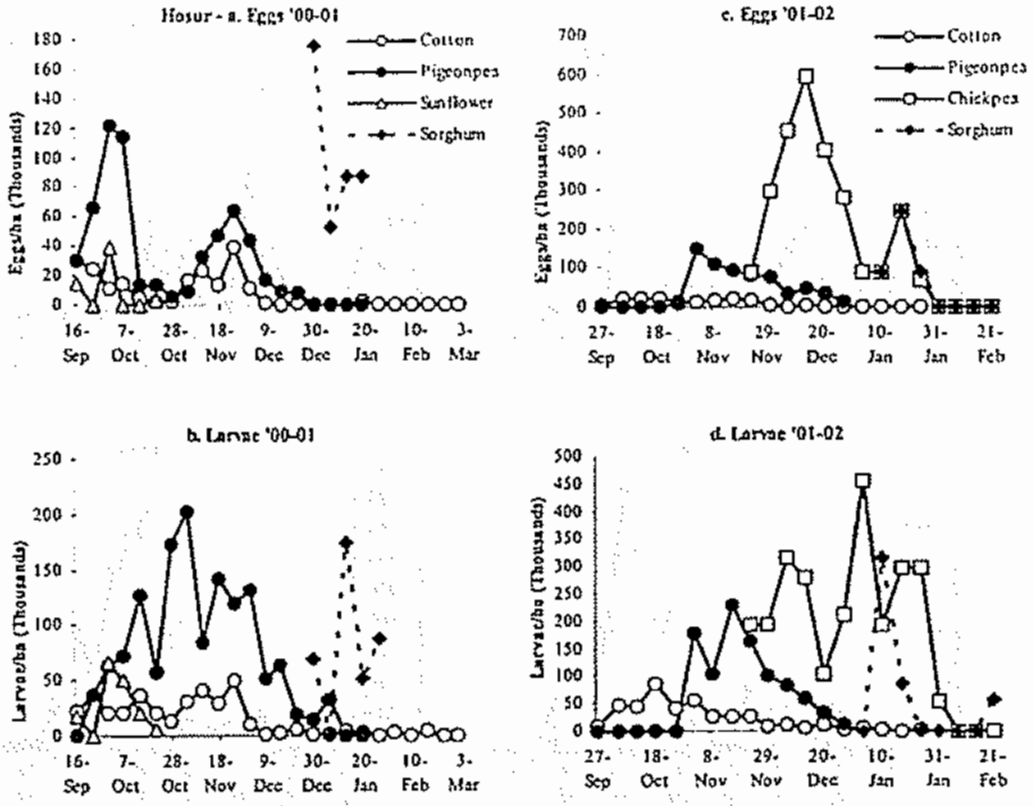


Fig. 7. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) during 2000–2001 and (c) eggs and (d) larvae during 2001–2002 at Hosur in Karnataka.

various host crops at different study locations for 2000–2001 and 2001–2002 are presented in Tables 2 and 3. Comparisons of the abundance of eggs and larvae on cotton relative to the other host crops are presented in Table 4.

Egg Numbers. Egg numbers varied greatly among the crops and across locations, and large fluctuations also were seen over the growing season with all crops in both years (Figs. 2–9). However, oviposition coincided mostly with the bloom period in each crop. Oviposition was essentially continuous on cotton and pigeonpea during the comparable time points at the Punjab Rao University farm, Wani Rambapur, Ekalashpur, Hosur, and the Tamil Nadu University farm in both years (Figs. 2, 3, and 6–8), with increased numbers of eggs during the bloom period. At Madanpatti (Fig. 9), continuous oviposition was observed on cotton and okra during both years, whereas oviposition was more sporadic on tomato. At Rangapur and Muttojipet during 2001–2002 (Figs. 4 and 5), continuous oviposition occurred on cotton and chili, but it was more sporadic on corn. At Ekalashpur and Hosur during 2000–2001 (Figs. 6 and 7), sorghum recorded the highest number of eggs at a single time for any crop, but the oviposition period was very short. Similarly, oviposition was relatively high on sunflower at the Punjab Rao University farm but only for a short period (Fig. 2).

Over all sites, egg numbers were significantly greater on pigeonpea, chickpea, and okra than on cotton at comparable times (Table 4). Indeed, at all locations where chickpea and okra occurred with cotton, the average number of eggs was higher on these crops than on cotton. The same was true of pigeonpea, except at the Tamil Nadu University farm where numbers were comparable. Chickpea recorded the highest average numbers, followed by sunflower and pigeonpea. Egg numbers on sunflower, tomato, chili, and corn were not statistically different from those on cotton.

Larval Numbers. Larval populations also fluctuated across the cropping period on all crops during both seasons (Figs. 2–9). Larval populations on cotton and pigeonpea were present almost throughout the season, with peaks coinciding with bloom periods at the Punjab Rao University farm, Wani Rambapur, Ekalashpur, Hosur, and the Tamil Nadu University farm (Figs. 2 and 3, and 6–8).

The larval population per hectare was greatest on chickpea followed by pigeonpea and was significantly greater than that on cotton in both cases (Table 4). Overall, larval numbers on sunflower, okra, tomato,

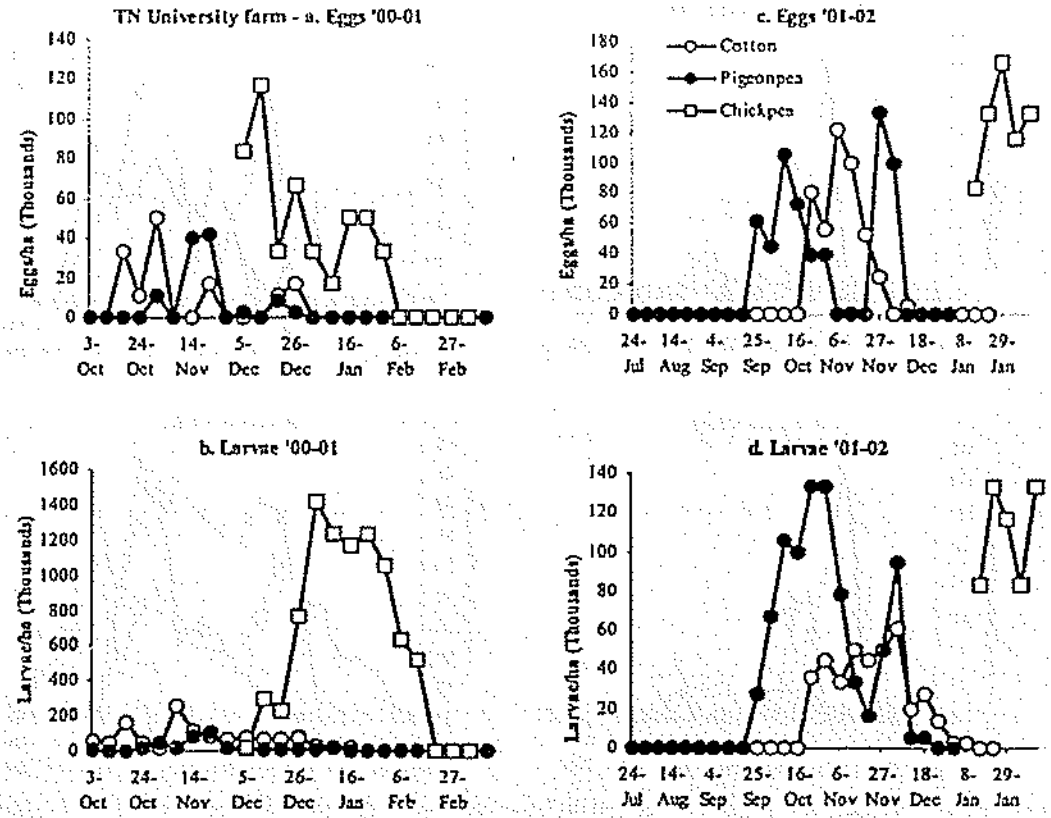


Fig. 8. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) during 2000–2001 and (c) eggs and (d) larvae during 2001–2002 on the Tamil Nadu Agricultural University Farm in Tamil Nadu.

and corn were not significantly different from cotton. Larval populations were more sporadic on these crops than on pigeonpea and cotton, but when populations were present, they tended to be higher on sunflower, okra, and tomato than on cotton (Figs. 2, 7, and 9). In contrast, larval numbers were consistently higher on cotton than on corn (Rangapur and Muttojipet, Figs. 4 and 5) and tended to be comparable or higher on cotton than chili (Wani Rambapur, Rangapur, and Muttojipet, Figs. 3–5). Overall, larval numbers were significantly lower on chili than on cotton (Table 4). Similarly, despite the heavy oviposition on sorghum at Ekalashpur, larval populations were low on sorghum in both years at this site (Fig. 6). At Hosur, sorghum supported significant larval populations but only for a short time (Fig. 7).

Discussion

H. armigera is a polyphagous insect pest in the Indian cropping system and completes ≈10 generations per year among the crops it attacks. The main aim of this 2-yr study was to evaluate the abundance of *H. armigera* on its major alternative host crops relative to cotton in central and southern India. The land holdings in these regions are small, with an average area of each crop of ≈1 ha ≈2–3 acres per farm. The host crops are grown in proximity to one another and pest movement among crops tends to be high.

Relative Abundance of *H. armigera* on Various Host Crops. As in other published studies, differences in the density of eggs on various hosts were seen in this study, possibly reflecting adult oviposition preferences. Chickpea had the highest density of eggs of *H. armigera*, followed by pigeonpea. Tomato and okra also had higher egg densities than cotton, whereas chili had lower egg densities than cotton. Sorghum also had much higher egg densities than cotton when eggs were present, but the crop duration was short and it was not present throughout the cotton-growing season. The abundant weed *L. mollis* also supported high densities of *H. armigera* eggs. In a previous comparable study, more eggs were recorded on tomato, sorghum, corn, and beans than on cotton (Parsons 1940). Similarly, studies conducted at Lam Farm, Guntur (India), indicated heavy oviposition on cotton, pigeonpea, tomato, and chickpea (Anonymous 1994). Laboratory studies on relative host preferences of *H. armigera* have shown cotton to be a less preferred host (Firempong and Zalucki 1990, Jallow and Zalucki

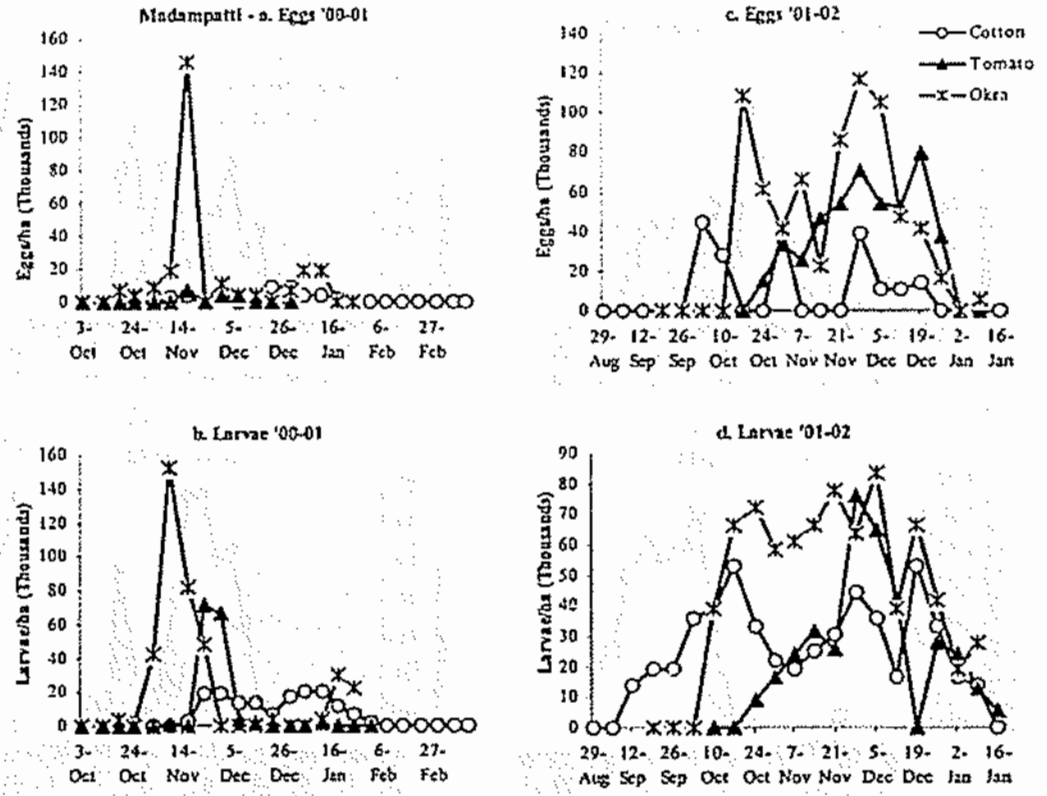


Fig. 9. Abundance of *H. armigera* (a) eggs and (b) larvae (in thousands per hectare) during 2000–2001 and (c) eggs and (d) larvae during 2001–2002 at Madampatti in Tamil Nadu.

1996). In addition, Ahrekar et al. (1999) also recorded large numbers of eggs of *H. armigera* on *L. mollis*. In general, ovipositional preferences have been attributed to the differential effects of microclimate, host plant volatiles, and other factors (Anonymous 1994, Jallow and Zalucki 1998, Cunningham et al. 1999, Jallow et al. 1999, Maezler and Zalucki 1999).

All major host crops supported larval populations in the current study. Both chickpea and pigeonpea supported significantly larger populations than cotton. Tomato and okra also had higher larval numbers than cotton, in agreement with studies conducted at Guntur on vegetable and field crops (Anonymous 1994). Similarly, in Australia, chickpea had the highest numbers of *H. armigera* larvae compared with other host crops (Miles and Ferguson 2001). In the current study, chili had lower larval populations than cotton. Although sorghum, sunflower and corn supported considerable larval populations, they also were relatively small compared with cotton and pigeonpea. In a previous study in India, corn was found to be an attractive host for *H. armigera* for oviposition, especially the

Table 2. Abundance of *H. armigera* eggs (mean ± SEM, in thousands per hectare) on various crops at eight sites over 2 yr

Site	Yr	Cotton	Pigeonpea	Chickpea	Sunflower	Sorghum	Okra	Tomato	Chili	Corn
PR farm	2000–2001	22.5 ± 9.1	26.8 ± 11.3		72.0 ± 12.0					
Wani Rambapur	2000–2001	10.3 ± 4.9	12.3 ± 4.4						6.5 ± 3.6	
Rangapur	2001–2002	39.8 ± 23.4							18.0 ± 6.6	13.7 ± 4.0
Muttojipet	2001–2002	11.9 ± 4.6							2.6 ± 1.0	5.4 ± 1.3
Ekalashpur	2000–2001	8.6 ± 2.8	31.6 ± 9.1	16.7 ± 25.6	0	0				
	2001–2002	14.8 ± 5.5	34.8 ± 19.2	52.5 ± 21.4	19.5 ± 19.2	0				
Hosur	2000–2001	7.9 ± 2.3	31.2 ± 8.7		9.5 ± 15.6	101 ± 30.2				
	2001–2002	8.6 ± 2.0	46.1 ± 13.4	156 ± 54.1		60.0 ± 91.4				
TN farm	2000–2001	8.7 ± 3.4	6.7 ± 2.9	345 ± 100						
	2001–2002	23.3 ± 9.5	218 ± 8.5	127 ± 15.1						
Madampatti	2000–2001	1.8 ± 0.6					15.5 ± 8.9	1.3 ± 0.7		
	2001–2002	9.0 ± 3.1					42.3 ± 11.6	33.3 ± 6.8		

Table 3. Abundance of *H. armigera* larvae (mean ± SEM, in thousands per hectare) on various crops at eight sites over 2 yr

Site	Yr	Cotton	Pigeonpea	Chickpea	Sunflower	Sorghum	Okra	Tomato	Chili	Corn
PR farm	2000–2001	6.7 ± 3.1	25.6 ± 14.0		12.0 ± 13.5					
Wani Rambapur	2000–2001	29.8 ± 16.8	37.5 ± 20.2						6.5 ± 3.6	
Bangapur	2001–2002	5.1 ± 3.0							19.0 ± 6.6	13.7 ± 4.0
Muttojipet	2001–2002	6.1 ± 2.2							2.6 ± 1.0	5.1 ± 1.3
Ekalashpur	2000–2001	13.8 ± 3.1	166 ± 39.1	811 ± 231	16.5 ± 13.5	0				
	2001–2002	16.2 ± 4.2	35.5 ± 14.3	560 ± 92.0	39.5 ± 11.1	0				
Hosur	2000–2001	14.5 ± 4.1	77.5 ± 14.7		9.5 ± 15.6	101 ± 30.2				
	2001–2002	23.3 ± 5.7	69.0 ± 21.1	185 ± 38.6		57.5 ± 41.1				
TN farm	2000–2001	73.1 ± 16.3	24.2 ± 6.2	612 ± 148						
	2001–2002	20.3 ± 5.2	35.1 ± 9.8	110 ± 12.6						
Madampatti	2000–2001	7.1 ± 8.0					22.8 ± 10.2	9.6 ± 5.7		
	2001–2002	25.0 ± 3.5					24.1 ± 5.7	46.1 ± 7.5		

silks, but few larvae survived because of heavy egg parasitism by *Trichogramma* species (Manjunath et al. 1970). A few observations on *L. mollis* showed substantial numbers of larvae, confirming its potential as an alternative host. This weed has been recorded as a prominent host for *H. armigera*, especially during the off-season (Rajendran 2000).

All of these hosts play an important role in sustaining *H. armigera* populations. In Australia, studies have demonstrated that hosts such as sorghum, sunflower, and small areas of corn produce a large population of the pest that moves to cotton throughout the season (Wardhaugh et al. 1980). Furthermore, in Australia, pigeonpea, sorghum, and corn produce more than twice the population of *H. armigera* observed on cotton (Fitt and Tann 1996, Sequeira and Playford 2001). Studies conducted in South Africa revealed that both weeds and indigenous plants supported significant larval numbers of *H. armigera* compared with cotton, thus acting as refuge for Bt cotton in small-scale farming areas (Green et al. 2003). Wu et al. (2002) have shown that, in China, natural refuge exists in terms of crops such as corn, soybean, and peanut that support greater larval populations than cotton. In the United States, other heliothine pests of cotton, including *Helicoverpa zea* (Boddie) and *Heliothis virescens* (F.), have been found to extensively use many alternative crop and weedy hosts (Schneider and Cross 1999).

Phenology of *H. armigera* on Various Host Crops. The bloom period of cotton and pigeonpea overlapped at all of the locations tested in the current study. The bloom period lasted 5–7 wk and was marked by higher egg densities and larval infestation on both crops. It is well known that *H. armigera* oviposition is particularly heavy during the flowering stages of its hosts (Parsons 1940, Roome 1975, Broadley 1978, Wardhaugh et al. 1980, Topper 1987, Nyambo 1988). In India, pigeonpea is grown throughout the country and often is grown along with cotton; in many places, it is cultivated as an intercrop. Planting of both crops occurs at a similar time, dictated by rainfall patterns. Flowering in pigeonpea usually starts 4–5 wk after cotton and continues beyond the bloom period of cotton. Given the synchrony in bloom periods, *H. armigera* adult populations emerging from cotton and pigeonpea should mix to a very large degree.

In contrast, chickpea is cultivated as a winter crop toward the end of the cotton and pigeonpea cropping periods and has little phenological overlap with cotton. In chickpea, the infestation by *H. armigera* starts on the foliage before flowering. Once the reproductive structures start occurring, the larvae move to

Table 4. Comparisons of mean no. eggs and larvae of *H. armigera* (mean ± SEM, in thousands per hectare) on cotton and alternative host crops across all locations during comparable time periods over 2 yr

Crop	Eggs			Larvae		
	N[a]	Mean ± SEM	Wilcoxon[b]	n	Mean ± SEM	Wilcoxon
Cotton	97	19.65 ± 2.83	**	114	30.04 ± 3.86	**
Pigeonpea		55.73 ± 9.11			72.33 ± 8.91	
Cotton	25	2.91 ± 0.91	**	30	15.105 ± 4.42	**
Chickpea		136.12 ± 30.29			622.49 ± 105.6	
Cotton	21	25.01 ± 7.51	NS	19	22.66 ± 3.86	NS
Sunflower		69.89 ± 19.84			46.82 ± 16.89	
Cotton	26	8.60 ± 2.68	**	29	19.61 ± 2.86	NS
Okra		35.83 ± 8.47			21.93 ± 6.32	
Cotton	15	9.53 ± 3.11	NS	30	21.46 ± 2.61	NS
Tomato		25.31 ± 6.55			29.93 ± 5.91	
Cotton	44	34.69 ± 14.26	NS	38	17.23 ± 0.69	*
Chili		14.81 ± 4.11			5.95 ± 1.84	
Cotton	32	37.79 ± 19.52	NS	35	5.56 ± 2.33	NS
Corn		12.85 ± 3.02			5.42 ± 1.39	

[a] Number of comparable sampling weeks.
[b] **$P < 0.01$; *$P < 0.05$; NS, nonsignificant at $P = 0.05$.

these parts. In Australia, chickpea is being used as a spring crop, whereas pigeonpea is used as an autumn crop (Fitt 1989) and for managing resistance to insecticides (Miles and Ferguson 2001).

The other crop hosts of *H. armigera* also overlap in phenology with cotton to varying degrees, and, in combination, several alternative hosts always can be found supporting populations at the same time as cotton. Similarly, in Australia, Fitt (1989) noted that *H. armigera* populations may develop simultaneously on a number of hosts within a region and exploit a succession of cultivated and uncultivated hosts through the season.

Published studies indicate that *H. armigera* populations coming from different host crops at the same time are capable of intermating and producing viable progeny. Kvedaras et al. (2000) showed that the larval host plant does not significantly influence the chance of a female moth being mated, despite substantial variation in moth abundance among crops. Trap catch studies in China also have demonstrated the potential of moths emerging from different crops to interbreed (Wu et al. 2002). However, the ability of moths from different hosts to interbreed will depend on several additional factors, including the distance between the crops and extent of moth movement. *H. armigera* adults are capable of moving long distances (Riley et al. 1992). Overall, the probability of moths from different hosts mating with each other will be very high in India because of the diverse cropping systems, small landholdings, and *H. armigera* biology.

Insecticide Resistance Management. Several strategies have been developed for the management of resistance in target insects to transgenic Bt crops. One commonly used resistance management strategy is to require farmers to plant non-Bt crop refuges in combination with Bt crop fields. These refuges are expected to produce large numbers of susceptible pest insects, which can mate with any resistant individuals developing on Bt crops. This significantly delays the evolution of resistance in the insect pest. In countries such as the United States, where Bt cotton accounts for a considerable proportion of the total area under cotton, these structured refuges consist of conventional cotton varieties.

However, in China, structured refuges are not required for Bt cotton. Instead, the cropping pattern of small, diverse farms results in alternative host crops of *H. armigera* being routinely grown alongside Bt cotton fields, and these hosts provide a "natural refuge" (Wu et al. 2002). Field studies carried out by Wu et al. (2002) in China have demonstrated that corn, peanut, soybean, and common cotton can serve as natural refuge for Bt cotton in certain regions of China. In small-scale farming areas in South Africa where Bt cotton is cultivated, indigenous plants and various weed species serve as alternative hosts for pests of Bt cotton (Green et al. 2003). The absence of any cases of insect resistance to Bt crops in any country after up to 9 yr of intensive commercialization probably reflects, in part, the role played by alternative host crops and weedy species, particularly in a country such as China where adoption levels of Bt cotton are very high in some provinces, and no structured refuges are planted by farmers (Tabashnik et al. 2003).

As in China, the Indian agricultural landscape is highly fragmented and different host crops of *H. armigera* are cultivated alongside cotton. Satellite mapping studies of cotton growing regions in central and southern India indicate that these alternative host crops (particularly pigeonpea) of *H. armigera* make up a substantial portion of the land area (K.C.R. et al., unpublished data). Nevertheless, the Indian Government, in approving Bt cotton for commercial cultivation in 2002, stipulated the planting of a structured refuge of 20% non-Bt cotton around the perimeter of the crop chiefly as an insecticide resistance management strategy. The pest distribution data presented here, together with the satellite mapping data on cropping patterns, indicate that structured refuges for Bt cotton may not be necessary in the cotton belt of central and southern India; a variety of alternative hosts seem to support large *H. armigera* populations throughout the cotton-growing season. Thus, India may be able to follow China in allowing natural refuge to substitute for "structured refuges," as suggested by Khadi et al. (2003).

Acknowledgments

We thank T. Rangwala, Y. B. Srinivasa, K. S. Muthukrishnan, and the members of the Crop Protection Division (Monsanto Research Center, Bangalore, India) for technical input; the collaborative institutions for the facilities they extended; and two anonymous reviewers for insights.

References Cited

Ahrekar, S. K., S. A. Nimbalkar, and S. N. Tikkar. 1999. *Lagascea mollis*—an alternate host for *Helicoverpa armigera* (Hub.). Insect Environ. 4: 120.

Anonymous. 1994. Biological studies of *H.* armigera, pp. 25–28. Regional Agricultural Research Station, LAM Farm, Andhra Pradesh Agricultural University, India.

Armes, N. J., D. R. Jadhav, and K. R. DeSouza. 1996. A survey of insecticide resistance in *Helicoverpa armigera* in Indian subcontinent. Bull. Entomol. Res. 86: 499–514.

Broadley, R. H. 1978. Distributions of *Heliothis* spp. (Lepidoptera: Noctuidae) eggs and 1st instar larvae on preflowering flue-cured tobacco plants in North Queensland. Queensl. J. Agric. Anim. Sci. 35: 73–76.

Cunningham, J. P., M. P. Zalucki, and S. A. West. 1999. Learning in *Helicoverpa armigera* (Lepidoptera: Noctuidae): a new look at the behaviour and control of a polyphagous pest. Bull. Entomol. Res. 89: 201–207.

Directorate of Economics and Statistics. 2003. Agricultural statistics at a glance. http://agricoop.nic.in/stats.htm.

Firempong, S., and M. P. Zalucki. 1990. Host plant preferences of populations of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) from different geological locations. Aust. J. Zool. 37: 665–673.

Fitt, G. 1989. The ecology of *Heliothis* in relation to agro ecosystems. Annu. Rev. Entomol. 43: 17–52.

Fitt, G., and C. Tann. 1995. Quantifying the value of refuges for resistance management of transgenic *Bt* cotton,

pp. 77–83. In D. Swallow [ed.], Proceedings of the Australian Cotton Conference, Broadbeach, August 1996.

Green, W. M., M. C. de Billot, T. Joffe, L. van Staden, A. Bennett-Nel, C.L.N. du Toit, and L. van der Westhuizen. 2003. Indigenous plants and weeds on the Makhathini Flats as refuge hosts to maintain bollworm population susceptibility to transgenic cotton (Bollgard). Afr. Entomol. 11: 21–29.

Jairaj, S. 1990. The problem of *Helicoverpa armigera* in India and its integrated pest management, pp. 1–16. *In Heliothis* management. Proceedings of the National Workshop, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore.

Jallow, M.F.A., and M. P. Zalucki. 1996. Within and between population variation in host-plant preference and specificity in Australian *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Aust. J. Zool. 44: 503–519.

Jallow, M.F.A., and M. P. Zalucki. 1998. Effects of egg load on the host-selection behaviour of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Aust. J. Entomol. 46: 291–299.

Jallow, M.F.A., M. P. Zalucki, and G. P. Fitt. 1999. Role of chemical cues from cotton in mediating host selection and oviposition behaviour in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Aust. J. Entomol. 38: 359–366.

Jandel Corporation. 1993. Sigmastat 2.0 for Windows version 2.0. Jandel Corporation, San Rafael, CA.

Kaushik, V. K., V. S. Rathoda, and N. K. Sood. 1969. Incidence of bollworms and losses caused to cotton in Madhya Pradesh. Indian J. Entomol. 31: 175–177.

Khadi, B. M., V. N. Kulkarni, I. S. Katageri, H. M. Vamadevaiah, and M. Marulappanavar. 2003. Strengths, weaknesses, opportunities and threats analysis of *Bt* cotton in India. Paper presented at the World cotton conference, Cape Town, South Africa.

Kranthi, V. 1997. Insecticide resistance management strategies for central India. Central Institute of Cotton Research, Nagpur, India. Technical Bulletin.

Kvedaras, O. L., P. C. Gregg, and A. P. Del Socorro. 2000. Techniques used to determine the mating behaviour of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in relation to host plants. Aust. J. Entomol. 39: 188–194.

Maelzer, D. A., and M. P. Zalucki. 1999. Analysis of long-term light-trap data for *Helicoverpa* spp. (Lepidoptera: Noctuidae) in Australia: the effect of climate and crop host plants. Bull. Entomol. Res. 89: 455–63.

Manjunath, T. M., V. R. Phalak, and S. Subramanian. 1970. First records of egg-parasitations of *Heliothis armigera* (Hübner) (Lep., Noctuidae) in India. Tech. Bull. Commonwealth Inst. Biol. Control 13: 111–115.

Manjunath, T. M., V. S. Bhatnagar, C. S. Pawar, and S. Sithanantham. 1989. Economic importance of *Heliothis* spp. in India and an assessment of their natural enemies and host plants, pp. 197–228. *In* Proceedings of the Workshop on Biological Control of *Heliothis*: increasing the effectiveness of natural enemies. New Delhi, India.

Miles, M., and J. Ferguson. 2001. Trap-cropping: a fad or a useful *Heliothis* management tool. www.dpi.qld.gov.au/fieldcrops/6976.html.

Mohan, K. S., and T. M. Manjunath. 2002. *Bt* cotton—India's first transgenic crop. J. Plant Biol. 29: 225–236.

Nyambo, B. T. 1988. Significance of host-plant phenology in the dynamics and pest incidence of the cotton bollworm, *Heliothis armigera* Hübner (Lepidoptera: Noctuidae), in Western Tanzania. Crop Protection 7: 161–167.

Parsons, F. S. 1940. Investigations on the cotton bollworm, *Heliothis armigera* (Hb.). Part III. Relationships between oviposition and the flowering curves of food plants. Bull. Entomol. Res. 31: 147–177.

Rajendran, T. P. 2000. Historical perspective, present scenario and future strategies of cotton pest management. Ag Technologies (Pest Management). www.indiaagronet.com/indiaagronet/pest_management/CONTENTS/integrated_pest_management.htm.

Roome, R. E. 1975. Activity of adult *Heliothis armigera* (Hb.) (Lepidoptera: Noctuidae) with reference to the flowering of sorghum and maize in Botswana. Bull. Entomol. Res. 65: 523–530.

Riley, J. R., N. J. Armes, D. R. Reynolds, and A. D. Smith. 1992. Nocturnal observations on the emergence and flight behaviour of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in the post-rainy season in Central India. Bull. Entomol. Res. 82: 243–256.

Schneider, J. C., and D. Cross. 1999. Summer survey of tobacco budworm and cotton bollworm populations in delta and hills of Mississippi. 1999 Report: on Monsanto's responses to points in January 5, 2000 EPA letter, submission dated 28 January 2000.

Sequeira, R. V., and C. L. Playford. 2001. Abundance of *Helicoverpa armigera* (Lepidoptera: Noctuidae) pupae under cotton and other crops in central Queensland: implications for resistance management. Aust. J. Entomol. 40: 264–269.

Sharma, H. C. 2001. Cotton bollworm/legume pod borer, *Helicoverpa armigera* (Hübner) (Noctuidae: Lepidoptera): biology and management. Crop protection compendium. International Crops Research Institute for the Semi-Arid Tropics.

Tabashnik, B. E., Y. Carriere, T. J. Dennehy, S. Morin, M. S. Sisterson, R. T. Roush, A. M. Shelton, and J. Z. Zhao. 2003. Insect resistance to transgenic Bt crops: lessons from the laboratory and field. J. Econ. Entomol. 96: 1031–1038.

Topper, C. P. 1987. The dynamics of the adult population of *Heliothis armigera* (Hübner) (Lepidoptera: Noctuidae) within the Sudan Gezira in relation to cropping pattern and pest control on cotton. Bull. Entomol. Res. 77: 525–539.

Wardhaugh, K. G., P. M. Room, and L. R. Greenup. 1980. The incidence of *Heliothis armigera* (Hübner) and *H. punctigera* Wallengren (Lepidoptera: Noctuidae) on cotton and other host-plants in the Naomi Valley of New South Wales. Bull. Entomol. Res. 70: 113–131.

Wu, K., Y. Guo, and S. Cao. 2002. Evaluation of natural refuge function for *H. armigera* (Lepidoptera: Noctuidae) within *Bacillus thuringiensis* transgenic cotton growing areas in North China. J. Econ. Entomol. 95: 832–837.

Received 13 April 2004; accepted 3 November 2004.

INSECT RESISTANCE MANAGEMENT PLAN FOR BOLLGARD II ® COTTON IN INDIA

Executive Summary

The approval of Bt Cotton (Bollgard® cotton) in India came with a condition of planting at least five rows of non-Bt cotton or 20 % of the total sown area as a refuge to ensure maintenance of susceptible populations of bollworms (*Helicoverpa armigera)* as a resistance management strategy.

Bollgard cotton (known as Ingard® cotton in Australia) has been grown in the U.S. and Australia since 1996 and 1997, respectively, on an ever-increasing number of acres without any evolution of insect resistance to the Bt protein in these products. At the time these products were commercialized, it was believed that the large farms and relatively homogenous cropping system found in the U.S. and Australia would require farmers to plant structured refuge areas with conventional cotton to maintain susceptible insects. This resistance management approach was based on limited data on the contribution of alternative host plants. Recent reviews by EPA (U.S.) and TIMS (Australia) of data on the contribution of alternative hosts have brought a recognition that the natural refuge provided by alternative hosts plays a prominent role in delaying resistance evolution to Bt proteins.

In China where Bt cotton also has been grown since 1997 and where land-holdings are small and fragmented, farmers have never been required to plant cotton refuge areas. Major alternative host crops of *H. armigera*, such as corn, wheat and some vegetable crops, provide a natural refuge in cotton-growing areas (Wu *et al.*, 2001). In India, cropping patterns are as diverse and fragmented as China, and similar observations have been made on alternative host contribution. Data were generated on target pest biology and the agronomics of cotton demonstrating that a number of alternative host crops serve as natural refuge for *H. armigera* in cotton-growing areas, thereby acting as a sufficient resistance management strategy. These studies were conducted by scientists of State Agricultural Universities, Agricultural Research Institution, Project Directorate of Biological Control (I.C.A.R.) and Monsanto Research Center, Bangalore.

Considering the losses incurred by Indian farmers in planting 20% structured refuge for Bollgard cotton, various specific studies on the performance and mode of action of Bollgard II® cotton,

and the opinions of the scientific community, we strongly recommend that no structured refuge be required for Bollgard II cotton in India. The important reasons to not require structured refuge for Bollgard II cotton are:

1. The superior insecticidal properties of Bollgard II cotton – expression of two Bt proteins (Cry 1Ac and Cry 2Ab2) at high levels produces excellent bioactivity against bollworms and hence low survival of these pests. The two Bt proteins are complementary to each other, each with high levels of insecticidal activity against all of the major lepidopteran pests of cotton in India. Furthermore, these two proteins have different modes of action and thus the chance of target insect species developing resistance to both proteins is remote.
2. Presence of alternative host crops in substantial proportions in the cotton belts of Central and South India provide susceptible pest populations that are large enough to ensure the durability of a highly effective, two gene product like Bollgard II cotton. In addition, non-Bt varietal and hybrid cotton itself will serve as refuge.

In view of the two modes of action and the effective dose of Bollgard II cotton against the key target pests and the mixed cropping systems on fragmented land-holdings in India, there should be no requirement of structured refuge for Bollgard II cotton in India. This will help needy Indian cotton farmers reap the economical benefit of an additional 20% area of Bollgard II cotton.

The present document provides the details underlying this IRM strategy, including summaries of the scientific information available on cultivation of Bollgard II cotton in small land-holdings in India.

INSECT RESISTANCE MANAGEMENT PLAN FOR BOLLGARD II COTTON IN INDIA

1. INTRODUCTION

H. armigera is the primary focus of integrated pest management programs (IPM) in India because it is a major pest on several crops and has developed resistance to currently used insecticides (Armes *et al.*, 1996; Kranthi, 1997). *H. armigera* is susceptible to the Cry1Ac protein and, in this context, Bt cotton technology offers much improved control of *H. armigera.* Field studies conducted with Bollgard cotton in India prior to commercialization have shown effective control of bollworms, viz., *H. armigera*; spotted bollworm, *Earias vittella*; spiny bollworm, *E. insulana*; and the pink bollworm, *Pectinophora gossypiella* (Barwale et al, 1999; Mohan and Manjunath, 2002).

Based on past experience with insecticides, the question uppermost in the minds of academics, researchers and regulators is whether *H. armigera* will gain resistance to the *in-planta* expressed proteins once Bollgard and Bollgard II cotton are commercially grown over large areas. However, Bollgard cotton was commercialized in the U.S. in 1996 and has been grown on more than five million acres every year since then without the evolution of resistance among bollworms to *in planta* produced Bt protein. The main strategy for resistance management in the U.S. has been to require farmers to plant non-Bt cotton 'refuge' areas. However, recent data collected in the U.S. has demonstrated a significant contribution of alternative hosts and changes to current refuge requirements are being considered by the U.S. EPA. In China, where land-holdings are small and fragmented, a structured refuge has never been required. The cropping pattern in China allows other major alternative host crops of *H. armigera*, particularly corn and some vegetable crops, to be grown alongside cotton and serve as natural refuge (Wu *et al.*, 2001).

A number of transgenic Bt cotton (Bollgard cotton) hybrids, developed by Maharashtra Hybrid Seed Company (Mahyco), underwent multi-location field trials during the kharif seasons of 1999, 2000 and 2001. At the meeting of the Genetic Engineering Approval Committee (Ministry of Environment and forestry, Govt. of India), held on March 26, 2002, the agronomic and bio-safety data for these hybrids were reviewed and Bollgard cotton was officially approved for commercial cultivation in India. Bollgard cotton is the first transgenic crop to be approved by the Government of India for commercialization. The approval came with a stipulation to plant a refuge of 20% conventional cotton around each Bollgard cotton field to ensure maintenance of susceptible populations of bollworms as a resistance management strategy. The same was

followed while approving several Bollgard cotton hybrids of other seed companies viz. Rasi, Ankur and Nuziveedu during 2004 and 2005.

Pyramiding of insecticidal genes is an important IRM strategy and involves introducing more than one insecticidal gene into the same plant. From the earliest development of Bollgard cotton, it was anticipated that combining a second Bt gene such as Cry2Ab2 with Cry1Ac would provide increased activity and an expanded spectrum of insect control. The presence of two insecticidal proteins with different modes of action would further delay the development of resistance by target pests (Perlak et al., 2001). With this background, Bollgard II cotton was introduced in the U.S. and Australia and its efficacy has been remarkably higher than Bollgard cotton. Commercial introduction of 'two-gene' Bt cotton (Bollgard II cotton) in India at the earliest opportunity is an essential part of the IRM strategy for Bt cotton technologies.

This report describes a proposed IRM plan for Bollgard II cotton in India, developed from a comprehensive set of resistance management-related studies carried out since 1999. These studies were designed to collect local data on the insecticidal performance of Bollgard II cotton and the Bt proteins expressed in Bollgard II cotton and target pest biology and the agronomics of cotton-growing in India, thereby defining IRM strategies that are suitable for Bollgard II cotton grown under Indian conditions. In particular, studies were carried out to: (1) test the sensitivity levels of target pests to the two proteins Cry1Ac and Cry2Ab2; (2) determine the baseline-susceptibility of Indian populations of *H. armigera to* Cry1Ac and Cry2Ab2; (3) assess the relative populations of *H. armigera* on cotton and alternative host crops grown in the same regions during the same season; and (4) estimate the relative proportion on an acreage basis of cotton and alternative host crops in the cotton-belt of South and Central India (vegetative mapping using satellite data). These studies involved collaborations between scientists of State Agricultural Universities, Agricultural Research Institution, Project Directorate of Biological Control (P.D.B.C.), Central Cotton Research Institute (CICR), and Monsanto Research Center, Bangalore. This report also discusses how Bollgard II cotton can be incorporated into current integrated pest management programs for cotton.

2. Resistance Management Plan for Bollgard II Cotton in India:

Based on the results of specific local scientific studies conducted at various institutes, and the performance of Bollgard II cotton technology under field trial conditions in India, the following Resistance Management Plan is proposed for Bollgard II cotton in India:

2.1. Establishment of Sensitivity of Bollworms to Cry1Ac and Cry2Ab2 proteins:

Basic information on the sensitivity levels of major cotton bollworms (*H. armigera*, *E. vittella* and *E. insulana*) and the tobacco defoliator, *Spodoptera litura*, to the Bt proteins Cry1Ac and Cry2Ab2 was generated during 1999. All of the bollworm species were shown to be susceptible to both proteins. Furthermore, Cry1Ac and Cry2Ab2 have been shown to have different insecticidal modes of action, including binding to different receptors and forming different sorts of pores in the gut of susceptible insects, making the combination of these two proteins a highly effective resistance management strategy.

2.2. Establishment of baseline susceptibility levels for Cry1Ac and Cry 2Ab2 protein:

Several laboratories including the Project Directorate of Biological Control, an ICAR institute, have independently evaluated the susceptibility levels of *H. armigera* populations from different parts of India to the Cry1Ac protein used in Bollgard and Bollgard II cotton. Similarly, Central Cotton Research Institute (CICR), an ICAR institute, has evaluated the susceptibility levels of *H. armigera* populations from different parts of India to the Cry 2Ab2 protein in Bollgard II cotton. These studies will serve as reference for monitoring populations of *H. armigera* for signs of tolerance to either the Cry1Ac or Cry2Ab2 protein.

2.3. Optimum dose strategy:

Bollgard cotton produces Cry1Ac protein throughout the season. Currently the technology provider ensures that all of the hybrids are enter into the regulatory system express an optimum dose of Cry1Ac throughout the critical part of the cotton growing season. Similarly, adequate measures have been taken to ensure that Bollgard II cotton expresses optimum doses of both the Bt proteins (Cry1Ac and Cry2Ab2) throughout the critical part of the growing season.

2.4. Utilising natural refuge options:

Academic studies carried out for two years in the Central and Southern Zones of India indicated that several alternative crop hosts of *H. armigera*, including pigeonpea, tomato, okra, sorghum and sunflower, are present in these regions at the same time as cotton and that these crops support large populations of *H. armigera*, which provide unstructured (natural) refuge for this pest. Several of the crops, especially pigeonpea and chickpea, are preferred hosts of *H. armigera*

over cotton and support substantial populations of *H. armigera* (Ravi et al., 2004). Satellite imagery studies clearly showed that considerable proportions of alternative host crops (up to 50% of the cropped area) are grown along with cotton during the kharif in Central and South India. Based on the population turn-over and cropping patterns observed, these alternative host crops will act as 'Natural Refuges' for *H. armigera*, hence structured refuge for Bollgard II cotton should not be necessary in the cotton belt of Central and South India. Currently comparable studies on vegetative mapping patterns are underway in North India and similar results as Central and Southern India are expected in Northern India. Furthermore, non-hybrid varieties of cotton which constitute about 4.3 million hectares of the total 9.0 million hectares of cotton provide an additional and substantial source of refuge for bollworms. The varietal cottons are likely to be a continuous source of refuge as the introduction of biotechnology is currently limited to hybrids only.

For these reasons, and the cost that structured refuges place on farmers in the form of yield losses, it is recommended that there be no structured refuge requirement for Bollgard II cotton in India.

2.5. Monitoring and Remedial action Plan:

Monitoring of Bollgard II cotton utilization by farmers and surveillance is a vital part of an IRM plan because it provides early warning of pest adaptation. It is expected that areas with the greatest utilization of the technology will encounter more intense selection pressure and should be the main areas of focus for monitoring possible resistance development. One of the most effective ways to monitor for resistance development is to scout field performance. For this to be effective, active grower participation is important in providing feedback to the technology provider for detection of suspected *H. armigera* resistance problems.

The monitoring analysis will be done at two levels at appropriate laboratories that have the expertise to conduct more detailed analysis:

1. Regular/Routine monitoring: Pest populations collected from high risk and high Bollgard II cotton adoption areas will be subjected to bioassays using discriminatory doses of the Cry1Ac and Cry2Ab2 proteins.
2. Reactive monitoring: *H. armigera* feeding damage or survival in Bollgard II cotton will be investigated to determine the cause of the occurrence, i.e., whether it is increased

insect tolerance or unrelated factors, including plants not expressing Bt protein; larval movement from non-Bt plants or weeds; or extremely heavy pest pressure. Confirmed occurrence of rare resistant *H. armigera* will be analyzed.

By examining the level of susceptibility of the pest or its progeny in any of the above mentioned tests, adaptation can be assessed through comparisons with previously established baseline susceptibility values determined prior to the introduction of Bollgard II cotton. This will provide a basis for early detection of resistance so that IRM strategies can be modified, if necessary, and mitigation measures implemented.

2.6. Encouragement of Integrated Pest Management (IPM) practices:

In the present cotton scenario in India where insect resistance has reduced the efficacy of many currently registered chemical insecticides, Bollgard cotton has become a major component of integrated pest management (IPM) where the major emphasis is on environmentally friendly approach. Bollgard II cotton produces the Cry1Ac and Cry2Ab2 proteins throughout the plant at a level high enough to control most bollworms; this is not possible with other currently available microbial, chemical or physical control methods. In addition, populations of predaceous and parasitic insects are known to increase in Bollgard cotton fields due to the reduced application of broad spectrum chemical insecticides against bollworms. These biological control agents then aid in the control of bollworms as well as other pests of cotton. Bollgard II cotton will decrease broad spectrum insecticide use even further, resulting in additional biological control benefits. The combination of Bollgard II cotton and beneficial insects will provide a safe and environmentally compatible foundation for the implementation of various control measures for other cotton pests.

It should be remembered that Bollgard II cotton offers protection only against certain lepidopteran larvae. Other pests like aphids, thrips, whiteflies and other sucking insects that are not susceptible to Cry1Ac or Cry 2Ab will not be affected by Bollgard II cotton and should be controlled by IPM practices including the use of insecticides if necessary. Bollgard II cotton should not be viewed as a stand-alone measure for all the pests of cotton.

The technology provider is continuously working with key institutes in all cotton growing areas to develop and promote appropriate IPM packages with Bollgard II cotton hybrids as a key component.

2.7. Educational and training programs:

Some of the resistance management strategies, including development of baseline susceptibility data, pest monitoring, optimum dose and development of multiple gene products, will be implemented by the technology provider, while others including IPM practices will be implemented by growers. Therefore it is critical that growers understand the value of implementing appropriate IPM tactics, especially proper scouting techniques and ETL-based spraying decisions. Providing growers with all the information on what they need to do to maximise the longevity and value of Bollgard II cotton will be an important part of the IRM strategy.

To effectively communicate appropriate IPM and resistance management practices, a multi-level approach is being adopted, including a) providing educational slide sets on resistance management and Bollgard II performance to academic and extension personnel, b) tours and meetings for growers and extension personnel, c) symposia/workshops to discuss and disseminate the information, and d) cooperative research with academic and extension specialists. These efforts will help to ensure that the benefits accruing from Bollgard II cotton cultivation, and the durability of the technology, are maximised. These programs will also address the issue of spurious and illegal cotton seed being sold as Bt cotton.

3. Summary of Results of Insect Resistance Management (IRM) related studies

3.1. Establishment of sensitivity of Bollworms to Cry1Ac and Cry2Ab2 proteins

Cotton bollworms (*H. armigera, E. vittella and S. litura*) were collected in the field and mass produced in the laboratory for bioassays. Neonate larvae of all three bollworm species were tested for their sensitivity to solubilized Cry1Ac and Cry2Ab2 proteins using diet incorporation assays. Mortality and growth related parameters were recorded. Both *H. armigera* and *E. vittella* were found to be highly sensitive to Cry1Ac (LC_{50} (µg/ml) values were 0.96 and 0.26, respectively) while *S. litura* was insensitive to Cry1Ac (LC_{50}(µg/ml) >100 ppm). However, all three species were highly sensitive to Cry2Ab2, with LC_{50}(µg/ml) values of 2.68, 2.97 and 3.61

ppm for *H. armigera*, *E. vittella* and *S. litura*, respectively. Therefore these two proteins are complementary to each other in their spectrum of activity and combining these two proteins can provide increased bioactivity against bollworms with positive implications for resistance management (Perlak et al., 2001). Studies in the United States have clearly indicated that the combination of these two proteins provides superior control of lepidopteran pests leading to further reductions in insecticidal sprays and higher cotton production (Ridge et al., 2000).

Furthermore, the Cry1Ac and Cry2Ab2 proteins have been demonstrated to have distinctly different insecticidal modes of action, as reflected in the ability of Cry2Ab2 to control Cry1Ac-resistant strains of *H. armigera* and *P. gossypiella* (see Table 1). This means that bollworms will be significantly less likely to develop resistance to Bollgard II cotton, expressing both of these proteins, than to Bt cotton products representing only a single mode of action.

Table 1. Lines of evidence demonstrating that Cry1Ac and Cry2Ab2 have different modes of action (from the attached report by Head and Reding, 2001)

Line of evidence	Results observed for Cry1Ac and Cry2Ab2
Protein structure	Amino acid sequence and tertiary structure of Cry1Ac and Cry2Ab2 are very different
Characterisation of binding receptors	Distinct classes of receptors observed for Cry1Ac and Cry2Ab2 in all species tested
Characterisation of binding kinetics and pore formation	Size of pores and the kinetics of binding differ between Cry1Ac and Cry2Ab2
Control of Cry1Ac-resistant insects	Cry2Ab2 protein or Bollgard II cotton control Cry1Ac-resistant cotton bollworm, tobacco budworm and pink bollworm

3.2. Establishment of baseline susceptibility levels for the Cry 1Ac and Cry 2Ab proteins

The baseline susceptibility of populations of *H. armigera* collected from 10 locations in the cotton growing regions of India to Cry1Ac protein was studied through bioassays in 1999. The study was repeated with 14 populations of *H. armigera* (from the cotton belt of Central and South India) as a regulatory requirement in 2001. The two-season study was carried out by scientists at the Project Directorate of Biological Control (ICAR), Bangalore (Jalali et al., 2004).

The mean susceptibility (LC_{50}) of the ten *H. armigera* populations studied in 1999 study was 0.44 µg/ml of diet, with a five-fold range (0.14-0.71) across the populations. The 2001 study produced a mean of 0.24 µg/ml, with a five to six-fold range (0.11-0.60) across the populations. These two studies served to benchmark the baseline-susceptibility of *H. armigera* prior to the commercialization of Bollgard cotton in India.

It is now possible to monitor for the possible evolution of resistance to the Cry1Ac protein in populations of *H. armigera* with reference to these baseline data. If field resistance were to occur, LC_{50} values should jump at least 50-fold, and up to 10,000-fold, as seen in laboratory selection experiments (Ferre and Van Rie, 2002). These values are monitored every year at CICR.

Baseline data also has been generated for the spotted bollworm against Cry1Ac (Kranthi et al., 1999).

Similarly, CICR has established baseline susceptibility values for Cry2Ab2 protein with *H. armigera* populations collected from various locations. Field strains of *H. armigera* were collected during October-December 2004, from cotton fields at 25 sites in the three cotton zones of India (North - 8 districts; Central - 11 districts; and South – 6 districts). The larvae were tested in a diet incorporation bioassay with Cry2Ab protein (in the form of Bt-corn leaf powder that contained 3.936 mg Cry2Ab in 100 mg of the leaf powder). Results indicated that the geographical variability in *H. armigera* susceptibility levels to Cry2Ab was comparable to that observed with Cry1Ac. The LC_{50} values ranged from 6.0 to 28.6 µg Cry2Ab/mL of diet with a mean ± standard deviation of 14.58 ± 4.94 across the 25 locations.

3.3. Relative abundance of *H. armigera* on cotton and alternative host crops

H. armigera is highly polyphagous in its feeding habits. In addition to cotton, it is a major pest on many other economically important crops including pigeonpea (*Cajanus cajan*), chickpea (*Cicer arietinum*), tomato (*Lycopersicon esculentum*), chilies (*Capsicum annuum*), sunflower (*Helianthus annus*) and maize (*Zea mays*) (Manjunath et al., 1989). Among these, pigeonpea and chickpea are the most preferred hosts. In Central and South India, cotton is predominantly grown in smallholdings of 1-5 acres. The cropping pattern is such that alternative host crops like pigeonpea, chilies, tomato and maize are grown in adjoining/nearby fields to cotton during the

post-monsoon kharif season (May/June to Oct/Nov). Chickpea, being a winter crop, has an overlap with the boll-bursting phase of cotton.

In order to compute the relative population sizes of *H. armigera* on all of these crops, a number of field studies were conducted in the kharif of 2000-01 and 2001-02. The objective of the studies was to determine the relative population levels of *H. armigera* on cotton and alternative host crops grown in the same areas in the same season. Concurrently, a laboratory study was conducted to examine whether moths of *H. armigera*, reared from larvae collected from the respective host plants, could cross mate and produce viable progeny. The study was carried out for two seasons (kharif of 2000-01 and 2001-02) during the entire growing period of the crops at:

1. Dr. Punjab Rao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra (2000-01)
2. College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka (2000-01 and 2001-02)
3. Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu (2000-01 and 2001-02)
4. Nagarjuna Agricultural Research Development Institute, Warangal, Andhra Pradesh (2001-02)

The conclusions drawn from the studies were:

Cotton is cultivated in Central and South India alongside several host crops of *H. armigera*. The major alternative host crops are pigeonpea, chickpea, sunflower, chili, tomato, okra, corn, chili and sorghum. All of these crops were observed to attract moths for oviposition and support substantial larval populations of *H. armigera*.

Chickpea, pigeonpea, tomato and okra supported higher populations of *H. armigera* than cotton. The larval population was 4-35 fold higher on chickpea, 2-18 fold higher on pigeonpea, and two fold higher on okra, sunflower and sorghum. Tomato supported larval populations equivalent to those on cotton. The populations on chili and corn were half the size of those on cotton. The weed, *L. milis*, also supported considerable numbers of *H. armigera* eggs and larvae during the same season.

The local agricultural practices led to synchrony in bloom periods between cotton and pigeonpea, which is marked by high pest incidence. This suggests that pigeonpea is a good refuge crop for cotton from a resistance management perspective. Furthermore, pigeonpea is commonly used as an intercrop with cotton in some cotton growing areas in India. Among the major host crops of

H. armigera, chickpea is the only winter crop. Because chickpea is a highly preferred host, moth populations of *H. armigera* from other crops can be expected to migrate to chickpea to complete a generation.

Laboratory studies showed that *H. armigera* collected from a large stand of a pure crop and reared in the laboratory can successfully cross mate with moths reared on other crops, producing viable progeny. Indirect evidence from the investigation and published literature indicates that substantial cross-flow of *H. armigera* populations occurs between crops (Ravi et al., 2005).

3.4. Vegetative mapping of alternative host crops in the cotton belt of Central and South India

Having studied the relative 'pest turn-over' of each crop, it was important to estimate the relative areas of the alternative host crops versus cotton in the cotton belt of Central and South India. This indicates the extent of 'natural refuge' present along with cotton during the growing season.

In a study coordinated by Monsanto scientists in India during the year 2000 and conducted by M/s. Agrinet Solutions Limited, Hyderabad (a non-governmental organization specializing in remote sensing and Geographical Information Service solutions in agriculture), crop use patterns were surveyed and relative proportions of cotton and other alternative host crops of the bollworm, *H. armigera*, were computed in selected locations in Gujarat, Maharashtra, Andhra Pradesh and Karnataka through remote sensing.

<u>The conclusions drawn from the studies were:</u>

1. Cropping patterns varied from location to location, including the proportion of alternative host crops.
2. Major alternative hosts for *H. armigera* are grown along with cotton during the kharif in Central and South India on up to 50% of the cropped area.
3. Pigeonpea as a sole crop is the major alternative crop in Maharashtra, Karnataka, Andhra Pradesh and Gujarat (18-46%). Pigeonpea and cotton intercrop is dominant in Maharashtra (53-99%) and certain parts of Andhra Pradesh (16-65 %)
4. Green chili follows pigeonpea in abundance in Andhra Pradesh (15-25%)
5. The area under sole cotton ranges from 60-68%. In some regions, non-*Bt* cotton itself will act as a refuge where it is cultivated to a large extent.

The satellite imagery studies clearly showed that considerable proportions of alternative host crops (up to 50%) are grown along with cotton during the kharif in Central and South India. Based on the population turn-over and cropping patterns observed, these alternative host crops will act as 'Natural Refuges' for *H. armigera*, hence structured refuges will not be necessary for the highly effective, two-gene product Bollgard II cotton in the cotton belt of Central and South India.

Furthermore, non-hybrid varieties of cotton, which constitute about 4.3 million hectares of the total 9.0 million hectares of cotton, provide an additional and substantial source of refuge for bollworms. The varietal cottons are likely to be a continuous source of refuge because the introduction of biotechnology is currently limited to hybrids only.

For these reasons, and because of the cost that structured refuges place on farmers in the form of yield losses, it is recommended that no structured refuge be required for Bollgard II cotton.

3.5. Bollgard as a component of an Integrated Pest Management (IPM) package

In the present cotton scenario in India, where insect resistance has reduced the efficacy of currently registered chemical insecticides, transgenic Bt cotton will be a critical component of integrated pest management (IPM). Bollgard II cotton expresses the Cry1Ac and Cry2Ab2 proteins throughout the plant at adequate levels to control bollworms. Such consistently sustained control of bollworm caterpillars is not possible with other available control methods.

In addition, because of the specificity of the proteins, populations of predaceous and parasitic arthropods can increase in Bollgard II cotton fields due to the reduced number or absence of applications of broad-spectrum chemical insecticides against bollworms. These biological control agents aid in supplementary control of bollworms and sucking pests of cotton. The combination of Bt cotton and beneficial insects provides a safe and environmentally compatible foundation for the implementation of other pest management and resistance management practices.

NCIPM (National Center for Integrated Pest Management), an ICAR institute, conducted Farmers' participatory trials on 33.18 ha in the Nanded district of Central zone to evaluate the performance of the Bollgard cotton hybrid MECH 162 under IPM and compared it with conventional hybrids/ varieties grown with and without IPM. The results indicated that there

was a significant reduction in bollworm incidence, particularly *H. armigera* and pink bollworm (*Pectinophora gossypiella*), and a reduction in the damage caused by bollworm to fruiting bodies, in Bt MECH-162. Maximum damage was observed in conventional cotton, where 7 sprays of pesticides were made compared to only three in Bt MECH-162. Populations of sucking pests were also lower on the Bt hybrid compared to conventional cotton. Seed cotton yield (12.4q/ha) and net returns (Rs 16231/ha) were highest with Bt MECH-162. The conventional cotton under IPM recorded a yield of 7.1 q/ha and returns of Rs 10507/ha. The results clearly show that IPM in cotton was most effective with Bt MECH-162, and that this combination provided the highest net returns (Bambawale et al., 2004).

With Bollgard II cotton, the benefits will be even greater in terms of reductions in chemical insecticides and increases in natural enemy populations, resulting in further increased yields.

In India, the IPM inputs currently recommended and used against bollworms include:

- Sex pheromone traps to monitor or mass trap the moths
- Egg-parasitoid, *Trichogramma chilonis*, released to destroy the eggs
- Predaceous green lacewing, *Chrysoperla carnea*, released to prey on eggs and freshly hatched larvae, as well as on sucking pests like whiteflies, thrips and aphids
- Nuclear polyhedrosis virus (NPV)
- Neem-based pesticides
- Selective ETL-based use of chemical insecticides only when absolutely necessary.

Wherever Bollgard II cotton is grown, external application of Bt products should be avoided for IRM reasons. All of the other suggested control measures are compatible with Bollgard II cotton. These options may be used simultaneously or sequentially depending upon the requirements.

In another study conducted by NCIPM during 2004-05, Bollgard cotton in IPM mode without structured cotton refugia has shown significantly higher yields compared to other treatments, including one of Bollgard cotton with 20% refuge under farmer practice where an increase of 35% in yield was observed (Bambawale et.al., 2005). Thus, the current practice of cultivation of Bollgard cotton with 20% cotton refugia seems to neutralize the economic advantage of Bollgard cotton to some extent.

3.6. Education and awareness program on Spurious / Illegal Bt

The spread of spurious and illegal Bt cotton is being perceived as one of greatest risks to the longevity (from a resistance management standpoint) and continued performance of this technology. To address this issue, in collaboration with Central and State law enforcement / Administrative agencies, the technology provider has taken various initiatives at different stakeholder levels to disseminate knowledge about potential hazards of cultivating and aiding sales of spurious and illegal Bt cotton seeds. The key initiatives are listed as follows:

At Farmer Level:

Education campaigns by way of media messages, farmer meetings, training programs, direct mailers, information brochures and direct one-to-one contacts, with the focus being on explaining potential crop failure risks, resistance risks and legal risks.

At Trade Level:

Educational and awareness campaigns though media, information brochures, training on seed acts and EPA-related rules to highlight potential crop failure risks and other legal risks.

At Local Ag Administration Level:

Awareness campaign in association with District and State Administration machinery to clarify legal validity and risks associated with such practices. Detailed training sessions were also organized by District Administration to create awareness of the Environmental Protection Act and its provisions to control such activity.

3.7. Other Initiatives

Continuous and constant tracking of product performance to ensure sustainability of the product is a key focus of the technology providers. Hence, relevant studies will be conducted in collaboration with key academic institutes to delay the development of resistance by bollworms to Bollgard II cotton.

References cited:

Armes, N. J., D. R. Jadhav, and K. R. DeSouza. 1996. A survey of insecticide resistance in *Helicoverpa armigera* in Indian subcontinent. Bull. Entomol. Res. 86: 499-514.

Barwale, R. B., K. S. Mukharjee, and V. R. Gadwal. 1996. Transgenic cotton and biotechnological approaches in cotton improvement research: in *Handbook of cotton in India. Indian Society for Ctton Improvement* (eds) V. Sundaram, A.k. Basu, K.R. Krishna Iyer, S.S. Narayanan and T.P. Rajendran (Mumbai, Central Institute for Research on Cotton Technology) pp 246-255.

Cunningham, J. P., M. P. Zalucki, and S. A. West. 1999. Learning in *Helicoverpa armigera* (Lepidoptera: Noctuidae): a new look at the behaviour and control of a polyphagous pest. Bull. Entomol. Res. 89: 201-207.

Ferre, J., and J. Van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 47: 501-533.

Perlak, F. J., M. Oppenhuizen, K. Gustafson, R. Voth, S. Sivasupramaniam, D. Heering, B. Carey, R. A. Ihrig, and J. K. Roberts. 2001. Development and commercial use of Bollgard® cotton in the USA – early promises versus today's reality. The Plant J. 27: 489-501.

Gould, F. 1986. Simulation models for predicting durability of insect resistant germplasm: a deterministic diploid, two locus model. Environ. Entomol. 15: 1-10.

Jalali, S. K., K. S. Mohan, S. P. Singh, T. M. Manjunath, and Y. Lalitha. 2004. Baseline-susceptibility of the old-world bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), populations from India to *Bacillus thuringiensis* Cry1Ac insecticidal protein. Crop Prot. 23: 53-59.

Kranthi, V. 1997. Insecticide Resistance Management Strategies for Central India. Central Institute of Cotton Research, Nagpur, India. Technical Bulletin.

Kranthi, S., K. R. Kranthi, and N. V. Lavhe. 1999. Baseline toxicity of Cry1A toxins to the spotted bollworm *Earias vittella* F. Crop Prot. 18: 551-555.

Manjunath, T. M., V. S. Bhatnagar, C. S. Pawar, and S. Sithanantham. 1989. Economic importance of *Heliothis spp.* in India and an assessment of their natural enemies and host plants, pp. 197 - 228. *In*: King, E. G. and Jackson, R. D. (Eds). Proc. Workshop of Biological Control of Heliothis: Increasing the effectiveness of natural enemies. 11-15 Nov. 1985, New Delhi, India. Far Eastern Regional Research Office, U.S. Department of Agriculture, New Delhi, India, 550 pp.

Mohan, K. S., and T. M. Manjunath. 2002. *Bt* cotton-India's First transgenic crop. J. Plant Biol. 29: 225-236.

Ravi, K.C., K. S. Mohan, T. M. Manjunath, G. Head, B. V. Patil, G. P. Angeline Greba, K. Premalatha, J. Peter, and N. G. V. Rao. 2005. Relative abundance of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on different host crops in India and the role of these crops as natural refuge for *Bacillus thuringiensis* cotton. Environ. Entomol. 34: 59-69.

Ridge, R. L., S. G. Turnipseed, and M. J. Sullivan. 2000. Field comparison of genetically modified cottons containing one strain (Bollgard®) and two strains (Bollgard II®®) of *Bacillus thuringiensis kurstaki*. In *Proc. Beltwide Cotton Conf.* Volume 2 (Dugger, C.P. and Richter, D.A., eds). Memphis, TN: National Cotton Council of America, pp.1057-1058.

Roush, R. T., and J. A. McKenzie. 1987. Ecological genetics of insecticide and acaricide resistance. Ann. Rev. Entomol. 32: 361-380.

Roush, R. T. 1994. Managing pests and their resistance to *Bacillus thuringiensis*: can transgenic crops be better than sprays? Biocontrol Sci. Technol. 4: 501-516.

Wu, K., Y. Guo, and S. Gao. 2002. Evaluation of natural refuge function for *H. armigera* (Lepidoptera: Noctuidae) within *Bacillus thuringiensis* transgenic cotton growing areas in North China. J. Econ. Entomol. 95: 832-837.

J. Plant Biol., Vol. 29 (3), December, 2002, pp. 225–236.
© Society for Plant Physiology and Biochemistry

REVIEW ARTICLE

Bt Cotton – India's First Transgenic Crop

K. S. Mohan and T. M. Manjunath*

Monsanto Research Center, Chowdaiah Road, 18th Cross, Malleswaram, Bangalore 560 003, India

MS received 30 August 2002; accepted 14 November 2002

The Genetic Engineering Approval Committee (GEAC) of the Ministry of Environment and Forests, Govt. of India, on 26 March 2002, approved three of MAHYCO's (Maharashtra Hybrid Seed Co Ltd) *Bt* cotton (Bollgard®) hybrids – MECH 12, MECH 162 and MECH 184 – for commercial cultivation in India. This is a landmark decision as *Bt* cotton is the first transgenic crop to receive such an approval and, with it, India has made its long-awaited entry into commercial agricultural biotechnology. MAHYCO's *Bt* cotton hybrids contain Monsanto's 'Bollgard®' *Bt* gene, *cryIAc*. The *Bt* protein targets the cotton bollworm complex which includes the false American bollworm (*Helicoverpa armigera*), pink bollworm (*Pectinophora gossypiella*), spotted bollworm (*Earias vittella*) and spiny bollworm (*E. insulana*). This approval was preceded by a number of regulatory-guided laboratory studies and about 500 field trials were carried out from 1998 to 2001. These studies have demonstrated the bollworm-control efficacy and the benign characteristics, from an environmental and feed-safety perspective, of this technology. The primary benefit to the grower is through a reduction in insecticide usage on cotton as well as increased yield owing to effective bollworm control. Besides MAHYCO, the Indian Council of Agricultural Research, Agricultural Universities and several other public institutions were involved in these regulatory studies. The development of Bollgard® cotton, its ecological and economic benefits, safety, insect resistance management strategies and its current global status are discussed in this review.

Keywords: *Bacillus thuringiensis*, transgenic *Bt* cotton, Bollgard®, insect-protected crops, agricultural biotechnology, cotton bollworms, insect resistance management, integrated pest management.

Introduction

Cotton bollworms are responsible for heavy yield loss in cotton in India. Among the bollworms, the false American bollworm (*Helicoverpa armigera*) is the most dominant and destructive. Chemical insecticides are used extensively for the control of bollworms and other pests. The number of sprays may range from 5 to 15 or more with an all-India average of seven sprays per cotton cropping season. It is estimated that insecticides worth Rs 3000 crores (Rs 30 billion or US$ 600 million) are used annually in Indian agriculture, of which about Rs 1600 crores are spent for the control of cotton pests and of this Rs 1200 crores against bollworms alone! In terms of volume, about 54% of the total insecticides used in Indian agriculture is applied on cotton (Figure 1). This indicates the economic importance of bollworms in general and *H. armigera* in particular. Despite such huge efforts, pest control has not been effective mainly because a pest like *H. armigera* has developed resistance to many groups of insecticides including the synthetic pyrethroids, in many pockets of the cotton belt in India (Armes *et al.*, 1992, 1996; Kranthi, 1997). Thus, effective control of bollworms has become a challenge to Indian farmers, scientists and policy makers alike. In such a challenging scenario, it is hoped, based on widespread adoption and demonstrated success in other countries, bollworm-resistant *Bt* cotton, which has been approved by the Genetic Engineering Approval Committee, Govt of India, on 26 March 2002, will provide the much-needed succor to cotton farmers.

Bt cotton is a product of transgenic technology in plants. Its development can be traced to the seventies when rapid development of tools and methodologies in genetic engineering fuelled excitement and hopes of introducing 'useful' genes derived from sources across species barriers into crop plants. This technology was soon viewed as a harbinger of new advancements in medicine, industry, environmental-remediation, nutrition and agriculture. Huge investments in this new technology in the eighties and nineties saw products emerge out of the biotechnology pipeline. In agriculture, the initial promise has

*For correspondence. (e-mail: t.m.manjunath@monsanto.com)

now been realized by significant technical successes in plant genetic modification leading to the introduction of the first products of agricultural biotechnology into markets around the world. Some examples of the commercialized products are herbicide-tolerant corn, cotton, soybean and canola; virus-resistant papaya and squash; insect-tolerant corn, cotton and potato, to name a few. The estimated global area of transgenic crops in 2001 was 52.6 million ha (130 m acres). The increase in area between 2000 and 2001 was 19% equivalent to 8.4 m ha, which is twice the corresponding increase in area between 1999 and 2000 (James, 2001). The global status of transgenic crops, in general, in 2001 is depicted in Figure 2 and that of only *Bt* cotton in Table 1.

This review primarily looks at the development and experience of *Bt* cotton technology in countries where it was commercialized first and its value in addressing the cotton bollworm problem in India.

The progenitor of *Bt* technology (*Bacillus thuringiensis*)

B. thuringiensis (*Bt*) is a soil-inhabiting gram-positive bacterium with ubiquitous distribution. Many insecticidal proteins (δ-endotoxins) are synthesized by *Bt* during the sporulation phase. The insect-control value of these proteins has long been recognized (for review see Beegle and Yamamoto, 1992) and *Bt* spray formulations (a mixture of endospores and insecticidal crystals) have been in extensive use, since 1960, especially on horticulture and forest crops either as an alternative to chemical insecticides or as a supplement. *Bt* products, when applied as sprays, have certain limitations like difficulty in getting uniform coverage on the crop; can be easily washed away by rain or *Bt* proteins, on the plant surface, may get degraded by solar radiation, thus requiring repeated applications; need for labour and equipment etc. While the use of *Bt* as a spray formulation remains significantly behind chemical pesticides in agriculture, the long and environmentally benign use of *Bt* products has weighed in favour of *Bt* transgenic crops from a safety standpoint. Currently the number of characterized Cry (acronym for 'crystal' protein since in the bacterium the insecticidal proteins aggregate to form insoluble crystals) proteins included in the *Bt* toxin nomenclature is 248 (Crickmore *et al.*, 1998; 2002) sourced from more than 80 subspecies of *Bt*. This reflects the existing rich gene-pool of *Bt* insecticidal genes which can be potentially exploited in agriculture.

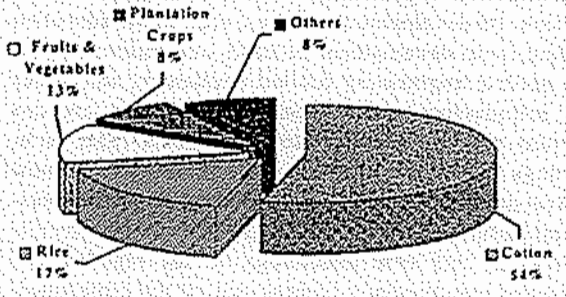


Figure 1. Crop-wise consumption of insecticides in India.

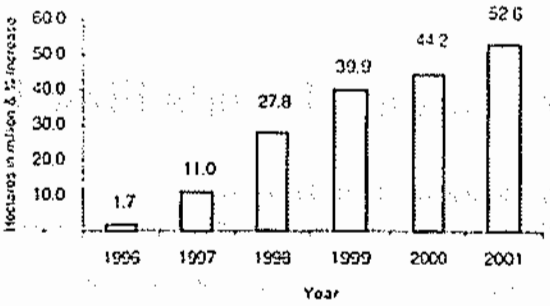


Figure 2. Global area of transgenic crops 1996–2001. Source: James (2001).

Table 1. Global status of Bollgard cotton 2001

Country	Year of approval	Area planted (in million)	
USA	1995	3.5 ha	8.4 acres
Australia	1996	0.5	1.2
Mexico	1997	0.5	1.2
China	1998	1.6	3.8
South Africa	1998	0.5	1.2
Argentina	1998	0.5	1.2
Indonesia	2001	0.05	0.1
Total		7.15 m ha (17.1 m ac.)	

towards collecting *Bt* isolates worldwide and screening them for novel insecticidal genes. Simultaneously, known *cry* genes and their products were subjected to molecular dissection in an attempt to improve the efficacy of spray formulations and hasten the drive towards *Bt* transgenic technology (Schnepf *et al.*, 1998).

Development of *Bt* cotton

An American cotton variety, Coker 312, was transformed with a gene construct containing a modified *cry1Ac* gene under the control of cauliflower mosaic virus 35S promoter containing a duplicated enhancer region. The development of *Bt* cotton progressed hand-in-hand with certain fundamental discoveries in gene expression in plants (reviewed by Perlak *et al.*, 2001). The levels of Cry1Ac in cotton flower buds (square) and green bolls (fruiting structures) were found to confer substantial protection from insect damage under conditions simulated for heavy insect infestation in the greenhouse (Wilson *et al.*, 1992; Perlak *et al.*, 2001).

In China, a biotechnology program for development of *Bt* cotton was started in 1991 at the Biotechnology Research Center of the Chinese Academy of Agricultural Sciences (CAAS) and the first genetically engineered cotton plant was produced in 1993 (Pray *et al.*, 2001).

Commercial ventures with insect-tolerant *Bt* crops

The year 1996 was a turning point in the annals of crop protection. Three pest-resistant transgenic crops were commercialized in the US with the due approval of the regulatory authorities. All three were insect-protected by *in-planta* expressed *Bt* protein. These were *Bt* corn for protection against the European corn borer (*Ostrinia nubilalis*), *Bt* cotton against cotton bollworm species (*Helicoverpa zea*, *Heliothis virescens* and *Pectinophora gossypiella*) and *Bt* potato against Colorado potato beetle (*Leptinotarsa decemlineata*). The transgenes were modified *cry1Ab* in corn (Carozzi and Koziel, 1997), modified *cry1Ac* in cotton (Perlak *et al.*, 1990) and modified *cry3A* in potato (Perlak *et al.*, 1993). Some of the considerations for incorporating insect-tolerance in these three crops were commercial value, mechanism(s) to capture value for the technology, gravity of pest-resistance to chemicals and for solving insect problems unmet by chemical insecticides (Roush, 1997).

Demonstration of high-level of insect tolerance in *Bt* cotton in glasshouse experiments paved the way for pre-commercial testing of the product. The earliest field trials were conducted with the transformed cotton plants expressing a truncated Cry1Ac protein. Such efficacy trials (multi-locational) indicated effective control of *H. virescens*, *P. gossypiella* and *H. zea* (Rummel *et al.*, 1994; Jenkins *et al.*, 1997). The mean tissue damage in *Bt* cotton was observed to be 2.3% in flowers and 1.1% in green bolls as against 23 and 12% respectively, in non-*Bt* control plants (Benedict *et al.*, 1996). In addition to insect tolerance, the effect of introgression of a foreign gene on agronomic traits as plant morphology, yield and lint quality was also studied. In all of these cases, it was determined that Bollgard® cotton was identical to the non-transgenic parental varieties. In matters relating to transgenic products in the US, the US Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA), regulate the process beginning from registration-related testing in the laboratory and field to the commercial release of transgenic products. In addition to these regulatory bodies, researchers at universities and other public-funded institutions were involved in the various efficacy trials of *Bt* cotton in the US. This promoted transparency in the regulatory procedures for the assessment of efficacy of *Bt* cotton against the bollworms and its safety to food, feed and the environment.

Pre-commercial multi-locational trials of *Bt* cotton over a number of seasons reconfirmed its excellent control of *H. virescens* (tobacco budworm) and good control of *H. zea* (cotton bollworm), especially in the post-bloom period. A few insecticidal sprays were occasionally required for control of *H. zea* during heavy pest pressure (Jenkins *et al.*, 1997). As efficacy screenings and field trials progressed, a single transformation event (Monsanto 531) was identified as a good source line for *Bt* cotton. All Bollgard® (registered product of *Bt* cotton in USA) varieties in the US are descendents of the 531 event (Perlak *et al.*, 2001). The scope of efficacy trials expanded as

the 531 event was backcrossed into several US varieties. Bollgard® cotton received the approval from the regulatory authorities for commercial cultivation in the US in 1996. Following commercialization in the US, Bollgard® was also introduced into Argentina (1997), Mexico (1997), Australia (1998), South Africa (1998), China (1998) and Indonesia (2001). In each country, it has undergone the prescribed regulatory trials for commercial approval. In China, prior to the introduction of Bollgard®, the Chinese Biosafety Committee approved four CAAS *Bt* cotton varieties for commercial use in nine provinces. Among the developing countries, China had the highest annual growth rate of transgenic crops with a tripling of its *Bt* cotton area from 0.5 to 1.5 m ha in 2001.

The experience with *Bt* cotton in the US at commercialization can be summarized as follows:

- Based on screening of hundreds of transformants for bollworm efficacy, a cotton transformational event 531 was selected as the progenitor of Bollgard® products in the US. This event could be backcrossed into a number of varieties without deterioration in insect control value. Expression of *cry1Ac* was stable in a wide variety of genetic backgrounds.
- Bollgard® provided very effective control of tobacco budworm and pink bollworm across the diverse cotton varieties and field locations in the US which also had diverse insect pressure. However, control of *H. zea* was not complete, especially in the post-bloom period and when insect pressure was high. A few sprays of insecticide were necessary at higher economic threshold levels (ETL). Incomplete control of *H. zea* was predicted earlier (Perlak *et al.*, 1990; Jenkins *et al.*, 1997) based on the susceptibility level of *H. zea* and the season-long concentration of cry1Ac protein in the reproductive tissues.
- Bollgard® products had little activity against larvae of beet armyworm (*Spodoptera exigua*) and fall armyworm (*S. frugiperda*).

Globally, *Bt* cotton with 6.8 m ha (13% area) occupied the third position among transgenic crops in 2001. The first being transgenic soybean (33.3 m ha, 63% of global area), followed by transgenic corn (9.8 m ha, 19% area) and transgenic canola occupied 2.7 m ha accounting for 5% area. From a trait viewpoint, herbicide tolerance (deployed in soybean, corn, cotton) has been the dominant trait (77% or 40.6 m ha of the global transgenic 52.6 m ha) followed by insect tolerance (15% or 7.8 m ha) and stacked genes for herbicide and insect tolerance in cotton and corn occupying 8% or 4.2 m ha (James, 2001).

Benefits from *Bt* cotton in the US and other countries

Reduction in insecticide application

In the US, conventional cotton requires heavy application of insecticides for the control of the tobacco budworm/bollworm complex, more frequently during heavy bollworm pressure. The effectiveness depends on factors like timing, application method and on the susceptibility of insects to insecticides. As against this, Bollgard® was found to provide effective protection against the bollworm complex (complete control of tobacco budworm and pink bollworm at all times and that of *H. zea* at moderate infestation), thus generally requiring no insecticidal applications for their control. There was a sharp decline in insecticide usage on Bollgard®, as revealed by the fact that in 1998 alone the reduction was about two million pounds of active ingredient (Benedict and Altman, 2000). Data on reduction in pesticide usage and economic benefit due to growing Bollgard® in the US have been reviewed extensively (Gianessi and Carpenter, 1999; 2001; Agnew and Baker, 2001; Falck-Zapeda *et al.*, 1999; 2000). Similar reduction in insecticide use has been reported from China, Mexico, South Africa, Indonesia and Australia (ISAAA, 2002).

Economic benefits

As is typical of economic returns in cotton, the financial benefit to a Bollgard® farmer is also dependent on (1) region-specific agronomic practices, (2) bollworm pressure in the season, (3) quality of crop management, and (4) market price of cotton. Bollgard® has been adopted over 1/3 of the cotton acreage in the US with a steady annual increase. Economic comparisons between Bollgard® and conventional cotton, across the varied agro-climatic growing areas in the US, have been well studied (Bryant *et al.*, 1999; Mullins and Mills, 1999; Cooke *et al.*, 2000; Karner *et al.*, 2000; Reed *et al.*, 2000; Seward *et al.*, 2000).

Primarily, a Bollgard® farmer benefits through a reduction in insect control costs (determined by bollworm pressure in the season) and an increase in yield (a minimum of 10%) owing to effective bollworm control. Monsanto studies tracked economic benefit since 1995 in 485 farms covering a variety of growing conditions (Perlak *et al.*, 2001). These studies indicated that Bollgard® cotton exceeded the economic return of conventional cotton even under low pest infestation level. This is because Bollgard® provided good control of bollworms, and since the infestation level was below treatment thresholds in conventional cotton or could not be detected in scouting, absence of insecticide spray led to crop damage to various degrees. This is termed as 'sub-threshold' protection by Bollgard®. Perlak *et al.* (2001) reviewed the average economic benefit of Bollgard®, based on independent studies from 1995 to 1999. The benefit worked out to be $49.80/acre which included the value of a 10% yield increase. Monsanto-sponsored trials gave an average Bollgard® advantage of $44.70, which included 7% yield increase. In general, the Bollgard® advantage in the various trials depended upon the level of bollworm infestation in the growing season.

Pray *et al.* (2001) have published a study on the impact of *Bt* cotton in China at the farm level. It was jointly conducted by the Center for Chinese Agricultural Policy, Beijing and Department of Agricultural, Food and Resource Economics of Rutgers University, USA. The objective of the survey was to evaluate economic returns, income distribution, environmental and health impacts in rural China where small farmers dominate agriculture. Farmers had adopted *Bt* varieties of the Chinese Academy of Agricultural Sciences (CAAS) and of Monsanto along with conventional cotton for comparison. Important conclusions from the studies were as follows:

- *Bt* cotton was adopted equally well by small and big farmers alike. The main economic benefit was in production cost. Farmers who grew popular *Bt* varieties reduced the costs of production by 20–23% over conventional cotton. The comparative production costs were computed based on cost of seeds, insecticides, labour and other inputs and yield of lint.
- Small farmers (< 1 ha) who grew *Bt* cotton gained twice the income per unit of land.
- Use of *Bt* cotton had substantially reduced the usage of insecticides and also reduced exposure of farm labourers to pesticides.
- The study also looked into the benefit distribution – between the growers and the seed companies. The benefits of growing *Bt* cotton primarily went to the farmers. About 82.5% of the *Bt* cotton benefits in 1999 from the adoption the CAAS *Bt* cotton varieties and 87% of the benefits by adopting Monsanto–Delta Pine *Bt* cotton went to the farmers.

Cotton and bollworms in India

Cotton is an important cash crop in India and plays a significant role in the national economy. As a commercial crop, it supports millions of Indians through cultivation, processing and trade and contributes to the export income by Rs 36,000 crores. India has the largest area under cotton (representing 20 to 25% of the global cotton area), which has fluctuated between 8 to 9 million ha in the last five years. Of this, about 80% of the area is planted with American cotton (*Gossypium hirsutum* and *G. barbadens*), of which hybrid and variety cotton share equal area while the remaining 20% is occupied by 'Desi' cotton (*G. herbacium* and *G. arborium*). Maharashtra, Gujarat, Andhra Pradesh, Karnataka, Madhya Pradesh, Punjab, Haryana, Rajasthan and Tamil Nadu are the important cotton-growing states. Despite India having the largest area under cotton in the world, it ranks only third in global cotton production after USA and China. The national average is 300 kg/ha against the world average of 580 kg/ha. Many factors have been identified as responsible for low yield and among them insects and diseases are the most important. More than 160 species of insect pests, which include sap-sucking insects, tissue borers and defoliators, have been reported to infest cotton at various stages of its growth, causing losses up to 60%. Among the insect pests, bollworms (tissue borers) are the most common and devastating, requiring major efforts to save the crop.

The cotton bollworm complex in India includes the 'Old world bollworm' or 'false American bollworm' [*Helicoverpa armigera* (Hubner)], pink bollworm [(*Pectinophora gossypiella*) (Saunders)], spotted bollworm [(*Earias vittella*) (Fabricius)] and spiny bollworm [*Earias insulana* (Boisduval)]. All these

are lepidopterans. The tobacco caterpillar [*Spodoptera litura* (Fab.)], also a lepidopteran, is a sporadic pest on cotton. Although predominantly a defoliator in certain years, it can also damage cotton bolls and squares when there is an outbreak.

Among the bollworms, *H. armigera* is the most dominant and difficult to control chiefly due to widespread insecticide resistance, prolific and multivoltine pattern of breeding and its polyphagous habit. This pest is a destructive feeder in the sense that a single larva can damage many squares and bolls in cotton or tomato fruits or pigeonpea pods. These attributes enable this pest to maintain year-round population and cause damage. *H. armigera* is ubiquitous in its distribution but limited to the old world, i.e. Europe, Asia, Russia, Africa, Australasia and the Pacific Islands. This species does not occur in the Americas. The species occurring in the Americas are *H. zea* (Boddie) and *H. virescens* (Fab.). Hence, reference to *H. armigera* as 'American bollworm' is misleading. To avoid such confusion, it is better to call *H. armigera* as 'old world bollworm' or 'false American bollworm'. More than 180 plant species (crops and weeds) have been recorded as hosts of *H. armigera* in India. Of these, besides cotton, about a dozen crops including chickpea, pigeonpea, tomato, okra, sunflower and chilies are the major host-crops (Manjunath *et al.*, 1989). Some of these major alternative host-crops are grown along with cotton in central and south India as a normal cropping practice. Studies have shown that the relative population levels of *H. armigera* eggs larvae and pupae on these alternative crops, especially chickpea and pigeonpea, are higher than that on cotton, underlining the important role these crops can play as 'natural refuge' from a *Bt* resistance management viewpoint. *H. armigera* was not considered a major pest of cotton prior to 1960. It was only in the late 1970s, with the introduction of the upland cotton varieties, that it switched to cotton to emerge as a significant pest.

E. vittella and *E. insulana* are the first to appear on young cotton plants and damage the apical shoot. The plant compensates the shoot-damage by promoting side branches as a result such plants appear 'bushy'. Flowering is usually delayed in such plants and yield is also compromised. *P. gossypiella* attacks later in the season during boll formation and is known to be devastating. The larvae enter green bolls and complete their life cycle within the boll.

Since dependable alternative control methods are not available for the control of *H. armigera*, farmers continue to spray insecticides repeatedly without commensurate benefit, thereby incurring heavy losses. In this kind of scenario, Bollgard®, on the basis of encouraging results obtained in other countries, has the potential to provide the much-needed relief to farmers from cotton bollworms.

Bollgard® breeding program in India

Realizing the economic importance of cotton bollworms and the advantages *Bt* cotton offers, MAHYCO (Maharashtra Hybrid Seed Co, Maharashtra) took the initiative in introducing this technology into India. The chronology of events leading to the development of Indian *Bt* cotton by MAHYCO has been described by Barwale *et al.* (1999) and Manjunath and Mohan (2001). These are summarized in Table 2.

MAHYCO is a leading seed company in India, which has developed several cotton hybrids suitable for different agro-climatic regions. Some of these promising hybrids have been converted into Bollgard® using the converted parental lines, obtained by crossing with the *Bt* gene donor parent received in 1996 from Monsanto, USA. Bollgard® hybrids were field tested in the kharif seasons of 2000 and 2001 for region-wise performance in terms of yield, *Bt* protein (Cry1Ac) levels in various tissues, impact on bollworms and non-target beneficial organisms, agronomic traits, etc. These studies were conducted as per guidelines laid out by the Department of Biotechnology (DBT). The levels of Cry1Ac in terminal leaves, of the various MAHYCO hybrids tested, contained much more than the concentration needed for effective control of neonates of *H. armigera*. The concentrations of Cry1Ac in the reproductive tissues (squares and green bolls) in the same hybrids were relatively lower but enough for effective control of *H. armigera* in the early phase of growth. Expressed levels of Cry1Ac in Bollgard® tissues declined with the age of the plant (Ghosh, 2001), an observation similar to the experience with Bollgard® varieties in the US (Greenplate, 1999).

Table 2. Development of Bollgard® cotton in India by MAHYCO.

1995 (March)	MAHYCO applied to DBT (Department of Biotechnology, Govt of India) for permission to import Bollgard® (*Bt* cotton) seeds from Monsanto Company, USA.
1996 (March)	With the approval of DBT, a nucleus stock of about 100 g of Bollgard® seeds was received by MAHYCO from Monsanto, USA. MAHYCO initiated crossing with Indian cotton breeding lines to introgress *cryIAc* gene. Forty elite Indian parental lines converted for *Bt* trait.
1996–1998	Risk-assessment studies conducted using *Bt*-cotton seeds from converted Indian lines 1996–1997 – Pollen escape studies – Aggressiveness and persistence studies – Biochemical analysis 1998 – Toxicological studies on ruminants (goats) – Allergenicity study on rabbits
1998–1999	Conducted multi-location field trials at 40 locations in nine states. Data submitted to RCGM (Review Committee for Genetic Manipulation), Ministry of Science & Technology, Govt of India.
1999–2000	Field trials were repeated at 10 locations in six states. Data submitted to RCGM.
2000	RCGM's recommendation to GEAC. July 2000 – GEAC gave approval to MAHYCO for large scale field trials in 85 ha and seed production in 150 ha.
2001	Kharif 2001 – Large scale field trials covering 100 ha and some trials under All India Co-ordinated Cotton Improvement Project of the Indian Council of Agricultural Research.
2002	On 26 March 2002, GEAC approved MAHYCO's three Bollgard® hybrids, viz. Mech 12, Mech 162 and Mech 184 for commercial cultivation in India This is the first approval of a transgenic crop in India

Data generated on these aspects were submitted to DBT/RCGM for examination. On the basis of these results, GEAC had given approval for commercial cultivation of *Bt* cotton, pronouncing it to be beneficial and safe. The approval came with the condition that the farmers have to plant a minimum of five rows of non-*Bt* cotton along the periphery of their *Bt* cotton fields or grow by the side to an extent of 20% of *Bt* cotton area, whichever is greater. The idea is that the non-*Bt* cotton will serve as a 'refuge' for the bollworms and is a strategy to prevent or delay the development of resistance by bollworms to *in planta* produced *Bt* protein.

In view of the satisfactory control of bollworms, seen in the various field trials in India, it is felt that this technology will benefit the Indian cotton farmer to a net amount Rs 5000 per acre by way of savings on insecticides and the productivity will double to 700 kg per ha (Jayaraman, 2002).

Some issues with *Bt* cotton

The major issues related to transgenic crops are bio-safety-related. These have been addressed by companies interested in commercializing transgenic crop plants and by governmental agencies charged with regulating the technology and products. These include potential for toxicity, food allergenicity, cross-pollination and effect on non-target organisms including biological control agents. The regulatory authorities in every country ensure safety in these areas before giving approval for commercial cultivation. With regard to *Bt* cotton, it has undergone and passed all the prescribed regulatory bio-safety studies, both in India and other countries.

In India, as per the direction of DBT, several studies related to bio-safety were conducted. Feed-safety studies of *Bt* cottonseed meal were carried out with goats, buffalos, cows, rabbits, birds and fish. The results revealed that the animals fed with *Bt* cottonseed meal showed no ill-effects and were comparable to control animals in the various tests. These studies were carried out at the Industrial Toxicological Research Institute, Lucknow; National Dairy Research Institute, Karnal; Central Institute for Fisheries Education, Mumbai; Central Avian Research Institute, Bareily; National Institute of Nutrition, Hyderabad and Govind Vallabh Pant University for Agriculture and Technology, Pantnagar. In short, the various feed-safety studies conducted showed *Bt* cottonseed meal to be substantially equivalent to the non-*Bt* counterpart. Studies were also conducted on the effect of leachate from *Bt* cotton plant on soil rhizosphere and non-rhizosphere microflora, soil Collembola and earthworms. The results showed no

difference between the soils obtained from *Bt* and non-*Bt* plants. The information generated on pollen dispersal has established that airborne pollen transmission is limited to a couple of meters and the risk of undesirable introgressive hybridization with related species is minimal. This is because *Bt* cotton hybrids are tetraploid in genetic composition whereas the nearest relative, being the local 'desi' cotton, is diploid and hence genetically incompatible for hybridization.

Forty field trials conducted during the 1998–99 cotton season and 10 trials during 1999–2000 clearly indicated that *Bt* cotton hybrids provided effective control of the bollworm complex in all the locations. Overall, insecticide applications targeted against the bollworm complex were reduced by 70 to 100% in *Bt* cotton hybrids when compared to conventional non-*Bt* hybrids. *Bt* hybrids also provided 14 to 60% higher yields compared to their non-*Bt* counterparts and other hybrids (Barwale *et al.*, 1999).

An important feature of the commercialization of *Bt* cotton in India and also in other countries has been the proactive steps taken in the development and implementation of approaches to prevent or delay the development of pest resistance to the *in-planta* produced *Bt* protein. This is in contrast to traditional practice for chemical insecticides. In addition, the companies involved in the commercial development of *Bt* cotton have been the leaders in development of these resistance management approaches. A number of such strategies have been developed based on experimentation and mathematical modelling. [See Fischhoff (1992), for a summary of strategies considered for *Bt* cotton.] Some of these are:

High dose (optimum dose)

This is based on *Bt* plants expressing high levels of *Bt* protein in their tissues so that susceptible bollworm larvae and those that are heterozygous for resistance will be killed. Pre-commercialization screens normally ensure that the selected plants express the optimum dose.

Refuge

A 'refuge' is plants without the *Bt* gene planted close to those with the *Bt* gene. The idea is that *Bt*-susceptible insects will feed and proliferate on non-*Bt* plants and will remain susceptible to *Bt* protein. When they mate with the scant few that have become resistant from surviving on the *Bt* crop, their susceptible genes will dilute any resistant genes in the overall gene pool. This has proved to be a very effective way of suppressing or delaying the development of a resistant race of insects. For the refuge strategy to be effective, the insects must emerge from the refuge at the same time as the resistant ones and be close enough to mate with resistant insects so that homozygous resistant forms are not produced. *Bt* cotton farmers in the US and Australia have adopted this method. The refuge crops can also be alternative hosts of the bollworms. In India, especially in the cotton belts of Central and South India, the farmers grow cotton along with pigeonpea, sunflower, tomato in the same season and chickpea towards the end of cotton crop. All these crops are alternative hosts for *H. armigera* and support substantial population of this pest, particularly pigeonpea and chickpea. In short, a 'natural refuge' is in place even if market penetration by *Bt* cotton happens in a big way. In the first few years, not all farmers are expected to adopt Bollgard® cotton and substantial area of non-*Bt* cotton will be available as refuge.

Stacking (pyramiding) of genes. There are several advantages in introducing a second *Bt* gene (whose protein has a different mode of action in the insect midgut) into Cry1Ac-cotton. Primarily the additive effect of both the *Bt* proteins will ensure greater lethality to bollworms. Secondly, the product might have an enlarged host range. Thirdly, the product is expected to check the spread of resistant insects, because even if the insect becomes resistant to the first *Bt* protein, the second would be a lethal surprise to a resistant insect. Monsanto has developed a two-gene product, named Bollgard® II, in which the second *Bt* gene is *cry2Ab2*. The host-activity of Bollgard® II (stacked with *cry1Ac* and *cry2Ab2*) in the US indicated that it had an additive effect on bollworms and also could control other lepidopteran pests like the armyworms, *S. frugiperda* and *S. exigua* against which Cry1Ac is not effective. Field trials of 2000 have proved that Bollgard® II provided increased protection against the key lepidopteran pests of cotton, resulting in better boll retention and consequent higher yields (Perlak *et al.*, 2001; Voth and Greenplate, 2001). In view of the improved con-

trol of bollworms by Bollgard® II, a further reduction in insecticide usage can be expected once Bollgard® II is commercialized in the US and other countries (Ridge *et al.*, 2000). Bollgard® II is awaiting approval for commercialization; data have been submitted to the regulators in the USA on food, feed and environmental safety and is expected to be commercialized in 2003. MAHYCO has recently initiated Bollgard® II breeding program in India with the collaboration of Monsanto and approval of DBT.

From a resistance management perspective, Bollgard® II is an improved product because the chances of bollworms gaining resistance to both the proteins are extremely small. Insecticidal sprays on Bollgard® may be necessary if the bollworm load crosses the economic threshold level for damage (the level is 20 larvae in 20 plants in India). This kind of situation is not anticipated in Bollgard® II because of higher cumulative levels of both insecticidal proteins. The second gene in cotton can also be from non-*Bt* sources as plant-based 'trypsin inhibitor' or 'amylase inhibitor', if such proteins with sufficient insecticidal activity against the target pests can be identified.

Integrated Pest Management

Integrated Pest Management (IPM) is strongly recommended in India, as in other countries, for management of bollworms and other cotton pests. However, as effective alternative to chemical insecticides is still not available, farmers continue to rely heavily on insecticides without getting satisfactory results. *Bt* cotton should be able to fulfill this need but it should not to be viewed as a stand-alone technology to manage all cotton pests. While *Bt* cotton can largely control the bollworm complex, sucking pests and other non-lepidopteran pests will have to be tackled by combining biological and other strategies. No doubt it can serve as one of the most dependable inputs in IPM. The unique feature about transgenic *Bt* technology is that it is compatible with other pest management tools like biological control, sex pheromones, botanical insecticides and even chemical pesticides. Combining various strategies may be the most effective approach to delay *Bt* resistance development in pest populations. Practice of IPM can diversify mortality sources so that resistant strains are not selected by a single mortality mechanism (McGaughey and Whalon, 1992).

Discussion and conclusions

With the approval of *Bt* cotton for commercial cultivation, India has signalled her commitment towards seeking biotechnological solutions for addressing agricultural problems. Control of bollworms on cotton has been a long-standing problem with no effective solution in India and other cotton-growing areas of the world. The development of transgenic insect-tolerant crops, expressing insecticidal protein(s) of *B. thuringiensis*, was seen as a biotechnological approach to combating important insect pests. In view of the economic importance of cotton and the seriousness of bollworm problem, Monsanto in the US developed *Bt* cotton for bollworm management which were approved for commercial cultivation in 1996 as 'Bollgard®' in the US. This technology was readily accepted by the US growers as evidenced by the steady growth of *Bt* cotton-acreage from 0.7 m ha in 1997 to 3.5 m ha in 2001. The subsequent years saw commercialization of *Bt* cotton in several countries in quick succession in view of the economic and environmental benefits resident in this technology. The global acreage of *Bt* cotton has been growing and in 2001 it was 6.8 m ha equivalent to 13% of the area with transgenic crops. It is at this juncture of global acceptance that India has approved *Bt* cotton.

Cotton is an economically important crop in India too but productivity has been seriously limited by insect pest problems. The false-American bollworm (*H. armigera*) is the most serious among the bollworms resisting effective control by insecticides. Realizing the importance of *Bt* cotton in India, MAHYCO (technology partner of Monsanto) initiated *Bt* hybrid cotton breeding program in India. MAHYCO developed several *Bt* cotton hybrids and field-tested them from 1999 to 2001 for bollworm-control efficacy, yield and other agronomic traits, as stipulated by DBT, Govt of India. Bio-safety issues about the Indian *Bt* cotton hybrids have been addressed in the last three years through a series of regulatory-stipulated studies on environmental and animal feed-safety, many of them conducted by public funded laboratories. All these studies established that *Bt* cotton was substantially equivalent to conventional cotton. After scrutiny of research data, the Genetic Engineering Approval Committee, Govt of India, approved commercial cultivation of *Bt* cotton

in March 2002 – India's first transgenic crop, empowering the Indian farmers to participate in the biotechnological progress in India.

An Indian *Bt* cotton farmer is expected to benefit from savings on reduced usage of insecticides, conservation of biological control agents leading to supplementary pest control and by increased yield due to reduced crop damage. The environmental benefits which *Bt* cotton is likely to bring, cannot be costed in purely monetary terms, but they are clearly substantial. An average Indian farmer is likely to have a net benefit of at least Rs 5000 per acre by growing *Bt* cotton. Cotton agriculture in China, dominated by small farmers, is very similar to the situation in India. The benefits experienced by Chinese cotton farmers from 1998 onwards can be reasonably extrapolated to the Indian situation.

Management of potential resistance to the *Bt* protein among bollworms in the post-commercialization period is an issue to be addressed on a long-term basis and several strategies are being evolved. The suggestion of GEAC to maintain a refuge cotton crop along with *Bt* cotton is a step in this direction. Studies have shown that the cotton belt of Central and South India has substantial 'natural refuge' for *H. armigera* in the form of many alternative host crops like pigeonpea, chickpea, sunflower, maize, chili and tomato. Some of these crops are grown alongside cotton and occupy a substantial area. It is noteworthy that *Bt* cotton has been grown on millions of acres in the US since 1996 and also in several other countries, and so far there has been no evidence of bollworms gaining resistance.

Bt cotton enters the history of plant protection at a critical juncture when the paradigm of chemical control is giving way to IPM. Today, IPM is in need of a technology that can match the temporal efficacy of chemical insecticides while being safe at the same time. *Bt* cotton has the potential to fulfill this need and to bring significant value to growers, consumers and the environment.

Acknowledgements

The authors are thankful to their several colleagues in Monsanto and MAHYCO for their input during the preparation of this article.

References

Agnew C K and Baker P B 2001 Pest and pesticide usage patterns in Arizona cotton production; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) pp 1046–1054.

Armes N J, Jadhav D R, Bond G S and King A B S 1992 Insecticide resistance in *H. armigera* in South India; *Pestic Sci* 34 : 355–364.

Armes N J, Jadhav D R and DeSouza K R 1996 A survey of insecticide resistance in *Helicoverpa armigera* in Indian subcontinent; *Bull Entomol Res* 86 : 499–514.

Barwale R B, Mukherjee K S and Gadwal V R 1999 Transgenic cotton and biotechnological approaches in cotton improvement research; in *Handbook of cotton in India. Indian Society for Cotton Improvement* (eds) V Sundaram, A K Basu, K R Krishna Iyer, S S Narayanan and T P Rajendran (Mumbai, Central Institute for Research on Cotton Technology) pp 246–255.

Beegle C C and Yamamoto T 1992 History of *Bacillus thuringiensis* Berliner research and development; *Can Entomol* 124 : 587–616.

Benedict J H, Sachs E S, Altman D W, Deaton W R, Kohel R J, Ring D R and Berberich S A 1996 Field performance of cottons expressing transgenic Cry1A insecticidal proteins for resistance to *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae); *J Econ Entomol* 89 : 230–238.

Benedict J H and Altman D W 2000 Commercialization of transgenic cotton expressing insecticidal crystal protein; in *Emerging technologies in cotton breeding* (eds) J Jenkins and S Saha (Enfield, NH Science Publishers).

Bryant K J, Robertson W C and Lorenz G M 1999 Economic evaluation of Bollgard® cotton in Arkansas; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) vol 1, pp 349–350.

Carozzi N and Koziel M G 1997 Transgenic maize expressing a *Bacillus thuringiensis* insecticidal protein for control of European corn borer; in *Advances in insect control: The role of transgenic plants* (eds) N Carozzi and M G Koziel (London, Taylor and Francis) pp 63–74.

Cooke F T Jr., Scott W P, Meeks R D and Parvin D W Jr. 2000 The economics of *Bt* cotton in the Mississippi delta – a progress report; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) pp 332–334.

Crickmore N, Ziegler D R, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J and Dean D H 1998 Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins; *Microbiol Mol Biol Rev* 62 : 807–813.

Crickmore N, Ziegler D R, Schnepf E, Van Rie J, Lereclus D, Baum J, Bravo A and Dean D H 2002 *Bacillus thuringien-*

sis toxin nomenclature: *http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html*

Falck-Zapeda J B, Traxler G and Nelson R G 1999 Rent creation and distribution from biotechnology innovations: The case of *Bt* cotton and herbicide-tolerant soybeans in 1997; in *International service for the acquisition of agri-biotech applications briefs no 24* (New York, ISAAA).

Falck-Zapeda J B, Traxler C and Nelson R G 2000 Surplus distribution from the introduction of a biotechnology innovation; *Am J Agr Econ* 82 : 360–369.

Fischhoff D A 1992 Management of lepidopteran pests with insect resistant cotton: recommended approaches 1992; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, D J Herber and D A Richter (National Cotton Council of America) pp 751–753.

Ghosh P K 2001 Genetically engineered crops in India with special reference to *Bt*-cotton; *IPM Mitr* 1 : 1–21.

Gianessi L P and Carpenter J E 1999 *Agricultural biotechnology: Insect control benefits* (Washington, National Center for Food and Agricultural Policy).

Gianessi L P and Carpenter J E 2001 *Agricultural biotechnology: Updated benefits estimates*; (Washington, National Center for Food and Washington).

Greenplate J T 1999 Quantification of *Bacillus thuringiensis* insect control protein Cry1Ac over time in Bollgard® cotton fruit and terminals; *J Econ Entomol* 92 : 1377–1383.

ISAAA 2002 *Bt* cotton in China; *Bt* cotton in Mexico; *Bt* cotton in South Africa; *Bt* cotton in Indonesia; http://www.isaaa.org.

James C 2001 Global review of commercialized transgenic crops: 2001; in *International service for the acquisition of agri-biotech applications briefs no 24: Preview* (New York, ISAAA).

Jayraman K S 2002 India approves GM cotton; *Nature Biotechnol* 20 : 415.

Jenkins J N, McCarty J C Jr., Buehler R E, Kiser J, Williams C and Wofford T 1997 Resistance of cotton with endotoxin gene from *Bacillus thuringiensis* var. *kurstaki* on selected lepidopteran insects; *Agron J* 89 : 768–780.

Karner M, Hutson A L and Goodson J 2000 Bollgard® impact and value to Oklahoma's cotton industry 1996–99; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) pp 1289–1293.

Kranthi K R 1997 Insecticide resistance management strategies for central India; *Tech Bull* (Nagpur, Central Institute for Cotton Research).

Manjunath T M, Bhatnagar V S, Pawar C S and Sitanantham S 1989 Economic importance of *Heliothis* spp. in India and an assessment of their natural enemies and host plants; in *Proceedings of the workshop on biological control of heliothis: Increasing the effectiveness of natural enemies*, 11–15 Nov 1985, New Delhi, India (eds) E G King and R D Jackson (New Delhi, Far Eastern Regional Research Office US Dept. of Agriculture) pp 197–228.

Manjunath T M and Mohan K S 2001 *Bt*-cotton to combat bollworms: Its development and current status; in *Proceedings of the symposium on 'Biocontrol based pest management for quality crop protection in the current millennium'* 18–19 July 2001 (eds) S P Singh, B S Bhumannavar, J Poornima and D Singh (Ludhiana, Punjab Agricultural University) pp 173–181.

McGaughey W H and Whalon M E 1992 Managing insect resistance to *Bacillus thuringiensis* toxins; *Science* 258 : 1451–1455.

Mullins J W and Mills J M 1999 Economics of Bollgard® versus non-Bollgard® cotton in 1998; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) vol 2, pp 958–961.

Perlak F J, Deaton R W, Armstrong T A, Fuchs R L, Sims S S, Greenplate, J T and Fischhoff D A 1990 Insect resistant cotton plants; *Bio/Technology* 8 : 939–943.

Perlak J A, Oppenhuizen M, Gustafson K, Voth R, Sivasupramaniam S, Heering D, Carey B, Ihrig R A and Roberts J A 2001 Development and commercial use of Bollgard cotton in the USA – early promises versus today's reality; *The Plant J* 27 : 489–501.

Perlak F J, Stone, T B, Muskopf Y M, Peterson L J, Parker G B, McPherson S A, Wyman J, Love S, Reed G, Biever D and Fischhoff D A 1993 Genetically improved potatoes: Protection from damage by Colorado potato beetles; *Plant Mol Biol* 22 : 313–321.

Pray C E, Ma D, Huang J and Qiao F 2001 Impact of *Bt* cotton in China; *World Dev* 29 : 813–825.

Reed J T, Stewart S, Laughlin D, Harris A, Furr R and Ruscoe A 2000 *Bt* and conventional cotton in the hills and delta of Mississippi: Five years of comparison; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) vol 2, pp 1027–1030.

Ridge R L, Turnipseed S G and Sullivan M J 2000 Field comparison of genetically modified cottons containing one strain (Bollgard®) and two strains (Bollgard® II) of *Bacillus thuringiensis kurstaki*; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) vol 1, pp 1057–1058.

Roush R 1997 Managing resistance to transgenic crops; in *Advances in insect control: The role of transgenic plants* (eds) N Carozzi and M G Koziel (London, Taylor and Francis) pp 271–294.

Rummel D R, Arnold M D, Gannaway J R, Owen D F, Carroll S C and Deaton W R 1994 Evaluation of *Bt* cottons resistant to injury from bollworm; implications of pest management in the Texas southern plains; *Southwest Entomol* 19 : 99–207.

Schnepf E, Crickmore N, Van Rie J, Lereclus J, Baum J, Feitelson J, Zeigler D R and Dean D H 1998 *Bacillus thuringiensis* and its pesticidal crystal proteins; *Microbiol Mol Biol Rev* 62 : 775–806.

Seward R W, Shelby P P and Danehower S C 2000 Performance and insect control cost of Bollgard® vs conventional varieties in Tennessee; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) vol 2, pp 1055–1057.

Voth R and Greenplate J 2001 Bollgard® cotton technical review; in *Proceedings of the Beltwide cotton conference* (eds) T N Memphis, C P Dugger and D A Richter (National Cotton Council of America) vol 2, pp 830.

Wilson F D, Flint H M, Deaton W R, Fischhoff D A, Perlak F J, Armstrong T A, Fuchs R L, Berberich S A, Parks N J and Stapp B R 1992 Resistance of cotton lines containing a *Bacillus thuringiensis* toxin to pink bollworm (Lepidoptera: Gelechiidae) and other insects; *J Econ Entomol* 85 : 1516–1521.