

# Central Institute for Cotton Research, Nagpur.

## Annual Progress Report 2002-2003

### Monitoring for shifts in baseline susceptibility (development of tolerance/resistance) in the cotton bollworms (*Helicoverpa armigera*, and *Earias vittella* against Cry 1A(c) toxin in various cotton growing regions of the country'.

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## PART-1

### Baseline susceptibility of *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) to Cry1Ac

#### Introduction

Transgenic Bt-cotton crop was introduced into the Indian market in March 2002. One of the primary target pests of this technology in India and several other countries, is the cotton bollworm, *Helicoverpa armigera* (Hubner). *H. armigera* is a polyphagous pest with a wide host range of 181 plant species in India including cotton, maize, chickpea, pigeonpea, tomato, sunflower and several vegetable crops (Manjunath *et al.*, 1985). Insecticide resistance in *H. armigera* in India led to excessive and indiscriminate use of insecticides by desperate farmers in many parts of the country (Kranthi *et al.*, 2002). The introduction of insect resistant transgenic crops, especially *Bt* transgenic crops, is expected to be of immense value in pest management programmes. The technology is anticipated to result in effective control of lepidopteran pests and a significant reduction in the overall use of insecticides. However, long term exposure to the *Bt* transgenic crops is likely to render lepidopteran pests resistant to the Cry toxins due to continuous selection pressure. Insect populations that survive the concentration of toxin being expressed by a transgenic will be progressively selected for resistance over generations as a result of continuous exposure to the toxins. Moreover, the introduction of transgenic plants expressing Cry1A toxins under the influence of constitutive promoters is likely to

hasten the process of development of resistance. The development of resistance to *Bt* toxins can be quite distinct, depending upon the species, selection regimen or geographical origin of the founder colony (Heckel, 1994). Hence regular bioassays to assess the susceptibility of the test insect to the Cry toxins will monitor the changes in a baseline that can be used in monitoring resistance that may occur due to selection pressure of the Cry1Ac toxin. This report examines the changes in baseline toxicity, through detection of variability in the toxicity of Cry 1A toxins to *H. armigera* from different agroecological regions of India during 2002-2003 cropping season, which is the first year of Bt-cotton cultivation. Care was taken to ensure that insects were collected from regions in which Bt-cotton was cultivated.

## **Materials and methods**

### ***Preparation of Cry toxins***

The Cry1Ac protein was produced according to the methods in Albert *et al.*, (1990) from *Escherichia coli* strains containing hyper-expressing recombinant plasmid vectors pKK223-3 kindly provided by Dr Donald Dean, Ohio State University, US. The toxins were purified from over-expressing cells by sonication and extensive washing with 10 % sodium bromide. Proteins were quantified according to Lowry *et al.*, (1951) and the toxin was quantified on SDS-PAGE densitometry before preparing dilutions of six to ten concentrations in distilled water. The proteins thus produced contained 38 per cent of the full-length Cry1Ac toxin. Additionally, MVP-II (*Pseudomonas* encapsulated Cry1Ac 19.7% from Dow chemicals, USA obtained as a kind gift from Monsanto, India, Bangalore) was used for the bioassays. Because transgenic cotton produces the non-activated, full length Cry1Ac protein (~ 130 KD) (Sims *et al.*, 1996), the LC<sub>50</sub>, EC<sub>50</sub>, LC<sub>90</sub> and EC<sub>90</sub> values were determined for the full-length Cry1A proteins.

### ***Sampling regions and field strains***

Laboratory strains of *H. armigera* were established from larvae collected in cotton fields during the cropping season of 2002-2003 from major cotton growing regions India. Field strains of the cotton bollworm *H. armigera* were collected during September – October 2002, on cotton fields from 13 districts of central India (Nagpur, Akola,

amaravati, Yeotmal, Hingoli, Warora Beed, Jalgaon, Buldana, Aurangabad, Parbhani, Wardha and Nanded), 8 districts of North India (Hanumangarh, Sirsa, Fatehabad, Sriganaganar, Abohar, Mansa, Varanasi and Bhatinda) and 11 districts South India (Warangal, Khammam, Karimnagar, Nizamabad, Guntur, Darsi, Mancherial, Ongole, Adilabad Dharwad and Chirala). Efforts were made to collect insects from the regions where Bt-cotton was being cultivated. The strains were established on semisynthetic diet. A susceptible *H. armigera*, strain was established from isofemale lines at the CICR insectary and was used as a baseline susceptible strain for comparison (data not presented here).

Larvae were reared on a chickpea based semisynthetic diet (Armes *et al.*, 1992 ) individually in the 7.5 ml cells of 12 well 'ICN-Linbro' tissue culture plates till 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. Jars were kept at  $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and 70% R.H.

Strains were established for each field population from 100-200 moths and reared in the laboratory for three to four generations before conducting bioassays. Bioassays were carried out in 12-well 'ICN-Linbro' culture trays. 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 concentration of the toxins. A total of 5-6 concentrations of the toxins ranging from 0.005 to 5.0  $\mu\text{g/ml}$  diet were used for the bioassays. Mortality was recorded daily till the sixth day. Weight of the surviving larvae was recorded on the final day of observation. All assays were replicated two times and pooled data was subjected to analysis. The assays were performed in the laboratory at conditions of  $27 \pm 1^{\circ}\text{C}$  and 70% relative humidity. Median Lethal Concentrations ( $\text{LC}_{50}$ ) presented in table 1, were derived from log dose probit calculations (Finney, 1971) using POLO PC statistical package (Anon, 1987).  $\text{EC}_{50}$  values representing the effective concentration that prevent 50% of individuals in the treated population from reaching half the average weight of control larvae, were also derived from POLO-PC software and are presented in table 2. The treatment comparisons were made using the criterion of overlap of 95% fiducial limits according to Litchfield and Wilcoxin, (1949).

## Results & Discussion

The log dose probit response indicated that Cry1Ac was highly toxic to the bollworm larvae collected from all the sites in India (Table 1). Strains from south India were found to be more tolerant to Cry 1Ac compared to all other strains from rest of the country. The range of LC<sub>50</sub> was 0.017 to 0.27 in north India, 0.045 to 0.32 in central India and 0.042 to 0.540 µg Cry1Ac/mL of diet in south India. The variability in susceptibility across the strains was 32 fold, the most susceptible LC<sub>50</sub> value of 0.17 µg/mL from Sriganganagar and the highest value of 0.54 µg/mL from Adilabad. However the EC<sub>50</sub> range indicated a low variability in response with the difference between lowest (0.003 µg/mL of Wardha) and highest (0.043 µg/mL of Chirala) being only 14 fold. Three strains from north India (Mansa, Fatehabad and Varanasi), two from central India (Akola and Parbhani) and six from south India (Chirala, Warangal, Nizamabad, Guntur, Prakasam and Adilabad) showed tolerance levels that were higher (> 0.16 µg/mL) than the composite average (0.10 µg/mL) published baseline value (Kranthi et al, 2001). However, the tolerance observed herein was within the acceptable limits of the baseline, and did not indicate any shift in tolerance of *H. armigera* to Cry1Ac.

The fiducial limits (at 95% probability) of the probit assay data indicated that there was a good deal of variability in response of the different populations to Cry1Ac. The response of most of the field populations did not differ significantly from each other towards Cry1Ac as evident by an extensive overlapping of the fiducial limits. The  $\chi^2$  values indicated heterogeneity in response to the toxins in most of the field strains that were tested. This was not surprising, as this phenomenon has always been observed with all our earlier Cry1Ac bioassays with field strains of *H.armigera*. The reasons for such variability are not clear at this stage.

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. Resistance monitoring also enables the evaluation of the effectiveness of resistance management strategies. Traditionally, log dose probit assays, and recently diagnostic dose assays, have been routinely used to monitor development of insect resistance to insecticides (Forrester *et al.*, 1993; Kranthi *et al.*,

1997). The log dose probit assays are used to calculate resistance ratios ( $LC_{50}$  of the field strain/ $LC_{50}$  of the susceptible reference strain), whereas the diagnostic dose assays help to discriminate between resistant and susceptible phenotypes. Sims *et al.*, (1996) suggested that the most practical approach for dose validation was to use individuals sampled from numerous populations within the geographic range of the species. The data presented herein attempts to understand the significant differences in Cry1Ac susceptibility among *H. armigera* populations from different geographic locations within India.

Geographical variation in susceptibility to Cry1Ac through baseline susceptibility studies was earlier reported for *H. armigera* (Kranthi *et al.*, 2001; Wu *et al.*, 1999 and Fakruddin *et al.*, 2003) and the related species *H. virescens* and *H. zea* (Sims *et al.*, 1996).

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. The variability in toxicity was to an extent 32 fold to Cry1Ac. Compared to our (Kranthi *et al.*, 2001) earlier estimate of 67 fold, the current value seems to indicate a decreased variability in response of *H. armigera* to Cry1Ac. This is difficult to explain, as *Bt* sprays have not been extensively used in India except in integrated pest management (IPM) programmes carried out in Andhra Pradesh and Tamil Nadu by the State department agencies in intensively sprayed areas of Prakasam, Guntur and Coimbatore districts. Even then *Bt* sprays hardly constitute 0.1% of the total insecticides used on cotton in these districts. It would be interesting to examine if any other extraneous factors such as insecticides or cropping patterns were influencing the genetic variability in population response to the Cry toxins. Considering that *H. armigera* has a wide dispersal range and is migratory in nature, it is understandable that there were only marginal differences in susceptibility in populations that were collected from regions adjacent to each other. It was evident from the probit assay data that most of the field strains exhibited low to medium slopes in addition to a high level of heterogeneity within the populations. The highly mobile nature of the Heliothine species has always been considered as a factor making it difficult to interpret estimates of inter-population variation in *Bt* susceptibility (Fitt, 1989).

The introduction of *Bt* transgenic crops is an important addition to the existing

tools of integrated pest management. The technology is perceived to be effective and environmentally friendly. However, much of its success will depend on the sustained susceptibility of the target pests to the *Bt* toxins used in the transgenic crops. Differential expression in plant tissues may play an important role in the efficacy of the *Bt* transgenic crops. The current data show that one year of Bt-cotton cultivation in India has not contributed to any significant shift in the tolerance of *H. armigera* to Cry1Ac. If anything, it appears that the range of variability and the tolerance has declined in comparison to the published baseline (Kranthi *et al.*, 2001). It is important however, to ensure that appropriate Bt-cotton cultivation strategies must be designed to ensure the survival of susceptible insects and also ensure mating between the Bt-surviving and non-Bt-surviving insects. Such strategies have not yet been developed for the small farmer and predominantly un-irrigated cotton growing systems of countries such as India.

Table 1. Baseline susceptibility: Lethal concentration (LC<sub>50</sub>) of Cry1Ac to the cotton bollworm *Helicoverpa armigera*. Data of strains collected from thirty two cotton growing districts of India.

### NORTH INDIA

District	Collection date	n	LC <sub>50</sub> (95% FL)	LC <sub>90</sub> (95% FL)	Slope	± SE
Hanumangarh	September 02	240	0.061 (0.035-0.104)	0.386 (0.202-1.205)	1.59	0.16
Sirsa	September 02	240	0.045 (0.034-0.058)	0.190 (0.133-0.309)	2.04	0.22
Fatehabad	September 02	240	0.160 (0.080-0.385)	4.30 (1.30-52.235)	0.90	0.12
Sriganganagar	September 02	240	0.040 (0.029-0.054)	0.242 (0.162-0.419)	1.64	0.17
Abohar	September 02	264	0.146 (0.082-0.268)	0.721 (0.366-2.787)	1.84	0.20
Mansa	September 02	240	0.270 (0.130-0.66)	10.57 (2.92-35.87)	0.80	0.10
Varanasi	September 02	240	0.213 (0.150-0.314)	2.056 (1.149-4.896)	1.30	0.15
Bhatinda	September 02	240	0.057 (0.02-0.16)	3.04 (0.66-23.07)	0.74	0.11
Hanumangarh	November 02	240	0.069 (0.052-0.091)	0.328 (0.226-0.550)	1.89	0.20
Sirsa	November 02	240	0.033 (0.025-0.043)	0.130 (0.092-0.211)	2.15	0.24
Fatehabad	November 02	240	0.041 (0.031-0.053)	0.178 (0.125-0.293)	2.00	0.22
Sriganganagar	November 02	240	0.017 (0.013-0.022))	0.057 (0.041-0.090)	2.43	0.28
Abohar	November 02	240	0.082 (0.063-0.107)	0.351 (0.245-0.583)	2.02	0.22
Mansa	November 02	264	0.124 (0.085-0.186)	0.434 (0.272-0.977)	2.36	0.27
Varanasi	November 02	240	0.050 (0.03-0.080)	1.69 (0.75-6.14)	0.80	0.13
Bhatinda	November 02	240	0.118 (0.077-0.185)	0.528 (0.310-1.305)	1.97	0.21

### CENTRAL INDIA

District	Collection date	n	LC <sub>50</sub> (95% FL)	LC <sub>90</sub> (95% FL)	Slope	± SE
Nagpur	September 02	240	0.055 (0.042-0.071)	0.204 (0.146-0.329)	2.24	0.26
Akola	October 02	240	0.045 (0.035-0.058)	0.170 (0.121-0.276)	2.22	0.25
Amravati	October 02	240	0.105 (0.069-0.160)	0.501 (0.299-1.159)	1.88	0.20
Yeotmal	October 02	240	0.124 (0.090-0.172)	0.929 (0.582-1.797)	1.46	0.15
Hingoli	October 02	240	0.077 (0.045-0.130)	0.448 (0.241-1.317)	1.67	0.17
Warora	October 02	240	0.111 (0.070-0.180)	0.450 (0.258-1.236)	2.10	0.23
Nanded	October 02	240	0.046 (0.031-0.069)	0.173 (0.108-0.387)	2.24	0.26
Nagpur	December 02	240	0.096 (0.066-0.143)	0.377 (0.234-0.837)	2.16	0.24
Amravati	December 02	240	0.113 (0.064-0.208)	0.539 (0.276-2.00)	1.89	0.20
Yeotmal	December 02	240	0.047 (0.032-0.069)	0.160 (0.102-0.358)	2.40	0.29
Beed	December 02	240	0.062 (0.047-0.082)	0.288 (0.20-0.478)	1.93	0.20
Jalgaon	December 02	240	0.140 (0.094-0.212)	0.530 (0.325-1.217)	2.22	0.25
Buldana	December 02	252	0.101 (0.071-0.148)	0.320 (0.205-0.719)	2.57	0.32
Aurangabad	December 02	240	0.020 (0.016-0.026)	0.072 (0.052-0.114)	2.31	0.26
Akola	December 02	240	0.32 (0.22-0.480)	2.78 (1.52-7.090)	1.40	0.24
Nanded	December 02	264	0.118 (0.070-0.202)	0.483 (0.266-1.528)	2.09	0.23
Parbhani	December 02	240	0.180 (0.120-0.274)	2.53 (1.280-7.124)	1.10	0.13
Wardha	December 02	240	0.036 (0.023-0.057)	0.116 (0.071-0.305)	2.52	0.30

### SOUTH INDIA

District	Collection date	n	LC <sub>50</sub> (95% FL)	LC <sub>90</sub> (95% FL)	Slope	± SE
Chirala	October 02	240	0.348 (0.258-0.488)	1.969 (1.214-4.152)	1.70	0.21
Warangal	December 02	240	0.320 (0.22-0.510)	3.350 (1.710-9.457)	1.35	0.15
Khammam	December 02	240	0.130 (0.080-0.250)	7.563 (2.390-53.05)	0.74	0.11
Karimnagar	December 02	264	0.088 (0.056-0.141)	0.379 (0.219-1.001)	2.02	0.22
Nizamabad	December 02	264	0.170 (0.130-0.240)	1.200 (0.730-2.023)	1.64	0.16
Guntur	December 02	264	0.320 (0.160-0.750)	5.566 (1.79-27.850)	1.02	0.13
Darsi	December 02	240	0.260 (0.161-0.507)	2.603 (1.109-11.48)	1.30	0.16
Mancherial	December 02	240	0.030 (0.023-0.039)	0.119 (0.085-0.192)	2.16	0.24
Ongole	December 02	240	0.106 (0.081-0.139)	0.453 (0.316-0.750)	2.03	0.22
Adilabad	December 02	240	0.540 (0.340-1.020)	8.60 (3.40-20.903)	1.00	0.15
Dharwad	December 02	240	0.042 (0.032-0.054)	0.155 (0.112-0.245)	2.25	0.26

Table 2. Baseline susceptibility: Effective concentration (EC<sub>50</sub>) of the Cry1Ac to cotton bollworm *Helicoverpa armigera*. Data of strains collected from thirty two cotton growing districts of India.

### NORTH INDIA

District	Collection date	n	EC <sub>50</sub> (95% FL)	EC <sub>90</sub> (95% FL)	Slope	+ SE
Hanumangarh	September 02	240	0.009 (0.005-0.014)	0.126 (0.075-0.274)	1.13 ±	0.16
Sirsa	September 02	240	0.012 (0.008-0.018)	0.136 (0.084-0.281)	1.22 ±	0.16
Fatehabad	September 02	240	0.025 (0.016-0.037)	0.353 (0.206-0.780)	1.12 +	0.13
Sriganganagar	September 02	240	0.008 (0.003-0.014)	0.065 (0.033-0.264)	1.39 +	0.21
Abohar	September 02	264	0.013 (0.008-0.019)	0.14 (0.086-0.286)	1.24 +	0.16
Mansa	September 02	240	0.026 (0.017-0.038)	0.322 (0.192-0.679)	1.17 +	0.14
Varanasi	September 02	240	0.018 (0.008-0.034)	0.235 (0.108-1.04)	1.15 +	0.14
Bhatinda	September 02	240	0.010 (0.006-0.016)	0.178 (0.102-0.418)	1.04 +	0.15
Hanumangarh	November 02	240	0.005 (0.002-0.008)	0.061 (0.037-0.133)	1.17 +	0.19
Sirsa	November 02	240	0.007 (0.004-0.010)	0.057 (0.036-0.113)	1.42 +	0.22
Fatehabad	November 02	240	0.005 (0.002-0.009)	0.067 (0.041-0.146)	1.16 +	0.19
Sriganganagar	November 02	240	0.004 (0.001-0.006)	0.030 (0.019-0.063)	1.39 +	0.27
Abohar	November 02	240	0.005 (0.002-0.01)	0.041 (0.022-0.158)	1.43 +	0.24
Mansa	November 02	264	0.006 (0.003-0.01)	0.068 (0.042-0.144)	1.22 +	0.19
Varanasi	November 02	240	0.004 (0.002-0.007)	0.043 (0.027-0.092)	1.28 +	0.22
Bhatinda	November 02	240	0.008 (0.00400.012)	0.083 (0.051-0.174)	1.25 +	0.18

### CENTRAL INDIA

District	Collection date	n	EC <sub>50</sub> (95% FL)	EC <sub>90</sub> (95% FL)	Slope	+ SE
Nagpur	September 02	240	0.006 (0.002-0.011)	0.059 (0.032-0.200)	1.31 +	0.20
Akola	October 02	240	0.008 (0.003-0.013)	0.067 (0.036-0.236)	1.35 +	0.20
Amaravati	October 02	240	0.019 (0.007-0.037)	0.245 (0.106-1.41)	1.15 +	0.14
Yavatmal	October 02	240	0.010 (0.005-0.016)	0.196 (0.11-0.48)	0.98 +	0.14
Hingoli	October 02	240	0.018 (0.006-0.037)	0.27 (0.107-2.22)	1.08 +	0.14
Warora	October 02	240	0.018 (0.011-0.027)	0.241 (0.143-0.524)	1.14 +	0.14
Nanded	October 02	240	0.009 (0.004-0.014)	0.166 (0.094-0.405)	0.99 +	0.15
Nagpur	December 02	240	0.006 (0.004-0.010)	0.060 (0.038-0.124)	1.33 +	0.21
Amravati	December 02	240	0.007 (0.004-0.011)	0.068 (0.043-0.141)	1.31 +	0.20
Yavatmal	December 02	240	0.005 (0.003-0.008)	0.030 (0.20-0.060)	1.67 +	0.30
Beed	December 02	240	0.005 (0.002-0.008)	0.041 (0.026-0.084)	1.39 +	0.24
Jalgaon	December 02	240	0.006 (0.003-0.010)	0.058 (0.036-0.118)	1.33 +	0.21
Buldana	December 02	252	0.007 (0.002-0.013)	0.070 (0.035-0.299)	1.27 +	0.19
Aurangabad	December 02	240	0.005 (0.001-0.010)	0.046 (0.023-0.233)	1.35 +	0.23
Akola	December 02	240	0.013 (0.008-0.018)	0.113 (0.072-0.220)	1.35 +	0.17
Nanded	December 02	264	0.006 (0.004-0.009)	0.040 (0.026-0.077)	1.62 +	0.26
Parbhani	December 02	240	0.005 (0.003-0.008)	0.047 (0.030-0.098)	1.33 +	0.22
Wardha	December 02	240	0.003 (0.001-0.006)	0.026 (0.017-0.055)	1.45 +	0.29

### SOUTH INDIA

District	Collection date	n	EC <sub>50</sub> (95% FL)	EC <sub>90</sub> (95% FL)	Slope	+ SE
Warangal	December 02	240	0.013 (0.008-0.020)	0.164 (0.099-0.342)	1.18 +	0.15
Khammam	December 02	240	0.005 (0.001-0.009)	0.041 (0.022-0.159)	1.35 +	0.24
Karimnagar	December 02	264	0.005 (0.002-0.008)	0.042 (0.027-0.086)	1.37 +	0.23
Nizamabad	December 02	264	0.005 (0.002-0.008)	0.053 (0.033-0.114)	1.22 +	0.20
Guntur	December 02	264	0.013 (0.009-0.018)	0.093 (0.061-0.177)	1.49 +	0.19
Darsi	December 02	240	0.010 (0.006-0.015)	0.112 (0.070-0.231)	1.24 +	0.17
Mancherial	December 02	240	0.003 (0.001-0.005)	0.017 (0.011-0.035)	1.79 +	0.43
Ongole	December 02	240	0.007 (0.004-0.010)	0.061 (0.039-0.124)	1.35 +	0.21
Adilabad	December 02	240	0.018 (0.011-0.026)	0.208 (0.127-0.430)	1.20 +	0.15
Chirata	September 02	240	0.043 (0.029-0.063)	0.554 (0.321-1.22)	1.16 +	0.13
Dharwad	December 02	240	0.004 (0.002-0.007)	0.042 (0.026-0.090)	1.27 +	0.23

## PART-II

### **Baseline toxicity of Cry 1 Ac toxin against spotted bollworm, *Earias vittella* (Fab) using diet based bioassay**

#### **Introduction**

The spotted bollworm, *Earias vittella* (Fab.) is one of the bollworms attacking cotton during the early and mid- season of crop growth. It is important to generate baseline toxicity data of Cry 1Ac against this pest before Bt cotton causes shifts in pest tolerance and to enable detection of resistance development by the pest in the years to come. The current study employs a simple and reliable bioassay method based on semi-synthetic diet, to generate baseline data on the toxicity of Cry1Ac to *E. vittella* strains collected from eight cotton growing districts from Central and South India. Before generating the baseline, the diet was tested for its suitability by rearing spotted bollworm larvae continuously for at least six generations. The bioassay methods reported herein was standardized by replicated bioassays repeated on lab strains and its subsequent validation on sub- sets of F1 larvae from field populations.

#### **Materials and Methods**

Larvae were collected from cotton growing districts of South and Central India during October 2002. Of the 8 districts that were covered, 5 districts belonged to Andhra Pradesh, 3 districts to Maharashtra and 1 district to Karnataka. The districts covered were Nalgonda (1 site), Khammam (2 sites), Warangal (5 sites), Vijayawada (1 site), Adilabad (2 sites) Parbhani (1 site), Buldana (3 sites), Nagpur (1 site) and Dharwad (1 site). Bolls were brought from fields in muslin cloth bags and dissected out in the laboratory to recover larvae of *E. vittella*. Field collected larvae were reared on a wheat germ agar based semi- synthetic diet. The diet was poured out in multi-cell 12-well plates and larvae were reared singly from late second instar (approximately 6 days after hatching) onwards until pupation. Subsequent rearing of pupae and adults were similar to the protocol described earlier (Kranthi *et.al.*, 1999). Neonates were reared on tender terminal cotton leaves for two days before being transferred on to diet.

Cry1 Ac toxin protein was produced according to Albert and co-workers (1989) from *E. coli* strains containing hyper expressing recombinant plasmid vector pKK 223-3 kindly provided by Dr. Zeigler, Ohio State University, US. Toxins were purified from over expressing cells by sonication and extensive washing with 10 % sodium bromide. Proteins were quantified according to Lowry's method (1951) and diluted as six concentrations in distilled water.

A new bioassay method was designed keeping in view the low moisture requirement, of *E. vittella* neonate larvae. Cry1Ac was diet incorporated at six concentrations viz. 520, 104, 52, 10.4, 2.6 and 1.3 ng/ ml of diet. Diet was cooled to 55<sup>0</sup>C before addition of toxin. Toxin incorporated diet (10ml) was poured on to five filter paper strips of 1X0.5'' size, placed in petri plates and allowed to air dry in the laminar airflow. Strips coated with diet-incorporated toxin were placed individually in plastic cups of diameter 4.5 cm and height of 3 cm and 10 larvae of F1 generation were released per strip per cup. Cups were maintained at 27<sup>0</sup>C  $\pm$  1<sup>0</sup>C at 75 % R.H. Observations were recorded on alternate days for a period of 7 days. Toxin coated diet strips were also replaced on alternate days till the end of the experiment. Controls were maintained on plain diet strips. Bioassays were replicated at least thrice. Data were analyzed on POLO PC statistical package (Anon., 1987) for the determination of LC<sub>50</sub>s.

## **Results and Discussion**

The diet tested was found to be suitable for insect growth and did not involve the use of seed powder of either cotton or Okra (*Abelmoschus esculentis* Moench) as was reported earlier (Gupta *et al.*, 2000). Cost of the diet worked out to be \$10 per litre of diet that could support 3000 larvae of *E. vitella* in the younger instars and 720 third instar larvae for at least 3 days. Total time required for the development of larvae into pupae was 15.75 days that was slightly higher than on the most preferred host, Okra (11.64 days). The difference could probably be attributed to rearing of neonates on cotton leaves for the first 48h after hatching.

It is recommended that 6-day-old larvae of *E. vittella* be reared singly till pupation since they demonstrate cannibalistic behavior especially under conditions of over crowding or starvation.

LC<sub>50</sub>s obtained with Cry1Ac over three consecutive collections of *E. vittella* from Nagpur district (collections were made from the same site over September to December), using toxin incorporated diet strips were considerably replicable (Table3). The bioassay protocol is simple, as larvae do not require frequent handling, by transfer from one cup to another. Instead the diet strip could be replaced with fresh diet strips every alternate day. Spreading of 10ml of diet over five 1X0.5” filter paper strips ensured a relatively low moisture content in the diet as the surface area over which the diet was spread was high. This minimized larval feeding problems and bacterial contamination. Change of diet strip every alternate day is desirable to reduce probability of toxin degradation at prolonged exposure to room temperatures. Preliminary evidence indicates that this bioassay protocol also works well for pink bollworm, *Pectinophora gossypiella*.

Baseline susceptibility data is presented in Table 3. Toxicity of Cry1Ac ranged from 0.001 to 0.105 µg/ml of diet. The composite LC<sub>50</sub> value from 19 bioassays was found to be 0.024 µg/ml. Mortality response was clear four days after exposure to the toxin. Mortality increased between the fourth and seventh day of bioassay. Larvae that survived the toxin dose grew satisfactorily into pupation. Adult emergence and fecundity were as good as control although fertility seemed to be less than control especially in individuals treated with highest toxin dose. In fact this has been a persistent problem being encountered in our efforts to establish laboratory generated Cry1Ac resistant spotted bollworm.

Bt cotton is the first transgenic to be introduced in India. Resistance management programs have to be designed for the Indian situation with the available data. For resistance management programs to be effective, monitoring and surveillance for early detection of resistance are important. Resistance monitoring is important not only for the detection of any emergent resistant phenotype but also to determine the efficacy of resistance management strategies over time.

The efficacy of Bt transgenic cotton is also dependent on the variability in susceptibility to the Cry toxin in different populations across the country. The variability in toxicity of Cry1Ac to *E. vittella* was 105- fold. This is difficult to explain since Bt sprays have not been extensively used in cotton in India.

The LC<sub>50</sub> values of Cry1Ac when bioassays were conducted on cotton leaves were lower than the values obtained with the diet based bioassay method. The present LC<sub>50</sub> values also indicate that Cry1Ac is approximately 10 times more toxic to *E. vittella* compared to *H. armigera* wherein toxicity estimates for Cry1Ac are diet based for both insects. Keeping this in view it would be desirable to introduce indigenously developed Cry1Ac incorporated *Gossypium arboreum* transgenics in areas where this is an endemic pest.

It was interesting to note that at least a few individuals of some populations from Warangal, Vijayawada and Dharwad survived the highest concentration of the toxin. This suggests that under field situations tolerant individuals are likely to survive that may contribute directly to the resistance gene pool. Evidence towards this has been provided for *H. armigera* (Kranthi *et al.*, 2000) and resistance management strategies for Bt cotton have generally been targeted towards *H. armigera* with the premise that the two other bollworms are fully susceptible. This study emphasizes on the necessity for a management strategy for the bollworm complex that includes the three major bollworms of cotton.

Performance of at least eight Bt transgenics has been evaluated for the toxin production for two to three consecutive seasons in field situations (data partially published). Under Indian conditions the toxin levels dropped from 23 µg/g at 75 DAS (days after sowing) to 1-2 µg/g at 85 DAS in the hybrid MECH 162 (Kranthi, 2002). Differential expression of the toxin would reduce the efficacy of Bt transgenic crops. Spotted bollworm was collected in the current study from different sites at the boll bursting stage in South India (December 2002) and after the first pick in Central India. In South India the period of occurrence of *E. vittella* is between mid to late seasons of the crop when the toxin expression in transgenics would still be adequate for protection against this bollworm.

## Acknowledgements

We would like to thank the GEAC for ensuring that consistent efforts are taken to track down shifts in bollworm susceptibility to Cry1Ac. We extend our heartfelt thanks to Dr C. D. Mayee, Director CICR, who was responsible for CICR being identified as the central laboratory for baseline resistance monitoring. We thank Monsanto-Mahyco India for funding the project and providing MVP-II. Our sincere thanks to Dr Donald Dean, Ohio State University, US, for providing the Cry1A over-expressing clones.

Table 3. Cry1Ac baseline bioassay on the spotted bollworm *Earias vittella* (F1) collected from nine districts.

District	Collection date	n	*LC <sub>50</sub>	Fiducial limits	*LC <sub>90</sub>	Slope ± SE
Nalgonda	December 02	64	0.049	0.022-0.160	0.40	1.4 ± 0.5
Khammam	December 02	40	0.079	0.066-0.101	0.15	4.5 ± 1.0
Warangal	December 02	60	0.005	0.066-0.101	0.15	2.8 ± 0.4
	December 02	30	0.004	0.012-0.082	2.24	0.3 ± 0.4
	December 02	60	0.001	0.003-0.039	0.04	2.3 ± 0.3
	December 02	40	0.008	0.012-0.082	0.10	2.2 ± 0.3
	December 02	30	0.010	0.003-0.297	0.14	1.1 ± 0.4
Vijayawada	December 02	100	0.012	0.003-0.039	3.07	0.5 ± 0.1
	December 02	50	0.022	0.012-0.037	0.06	2.9 ± 0.5
Adilabad	December 02	40	0.049	0.160-1.040	0.50	1.4 ± 0.3
	December 02	50	0.105	0.054-0.557	0.78	1.5 ± 0.5
Dharwad	December 02	55	0.031	0.015-0.065	0.26	1.4 ± 0.4
Parbhani	December 02	30	0.036	0.066-0.101	0.15	1.0 ± 0.4
Buldana	December 02	50	0.036	0.012-0.267	2.61	0.7 ± 0.4
	December 02	50	0.025	0.012-0.060	0.20	1.4 ± 0.3
	December 02	50	0.020	0.006-0.089	0.20	1.3 ± 0.3
Nagpur	September 02	40	0.024	0.012-0.050	0.23	1.3 ± 0.4
	October 02	120	0.020	0.007-0.051	0.20	1.3 ± 0.3
	October 02	80	0.020	0.005-0.399	0.20	1.4 ± 0.2

\*LC<sub>50</sub>, LC<sub>90</sub> values are expressed as µg Cry1Ac incorporated per ml of diet

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To  
The Director,  
CICR, Nagpur

Sir,

Please find herewith four copies of the Annual Report 2002-2003 of the project Monitoring for shifts in baseline susceptibility (development of tolerance/resistance) in the cotton bollworms (*Helicoverpa armigera*, and *Earias vittella* against Cry 1A(c) toxin in various cotton growing regions of the country' for your kind approval to be submitted to the following. A copy may please be retained by the RCM unit, CICR, for record.

**Shri Desh Deepak Verma**

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Thanking you  
Yours Faithfully

**K. R. Kranthi**



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**This is to certify that the work reported herein as annual report of the project 'Monitoring for shifts in baseline susceptibility (development of tolerance/resistance) in the cotton bollworms (*Helicoverpa armigera*, and *Earias vittella* against Cry 1A(c) toxin in various cotton growing regions of the country' has been carried out during 2002-2003, at the Central Institute for Cotton Research under our supervision and we take full responsibility for the data being reported.**

14<sup>th</sup> July 2003  
Nagpur.

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