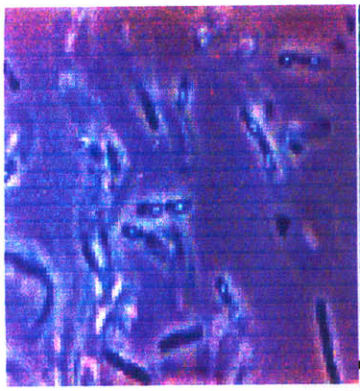
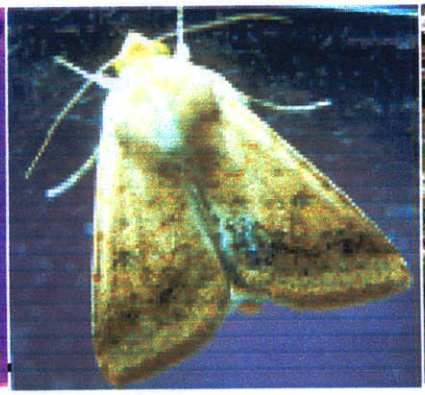


RESEARCH REPORT

(March 2005-Feb., 2006)

Of

the IARI Contract Scheme

entitled

"Baseline susceptibility of *Helicoverpa armigera* to Bt Cry1Ac toxin in as many as twenty selected different locations in India for monitoring resistance"

(No. 2-32(1)/ICC/2004 dt 15.12.2004)

Sponsored by

JK AgriGenetics Ltd., Hyderabad

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INTRODUCTION

Monitoring of insect resistance to *Bacillus thuringiensis* (*Bt*) Cry1Ac toxin is an essential component of resistance management in the *Bt* transgenic crops. It helps in discerning trends of development of resistance in insects both spatially and temporally. Such information could be used for developing strategies to delay development of resistance and thereby, sustain *Bt* transgenic crops as long as possible. *Bt* transgenic crops like cotton and corn are the only insect protective transgenic crops that were grown on about 15.7 million hectares and constituted about 19.5% of transgenic crop area in 2004 all over the world. Additional 6.8 million hectares of dual stacked *Bt* and herbicide tolerant corn and cotton constitute 8.5% of total transgenic area. Cumulatively, more than 80 million hectares have been cultivated under *Bt* corn and cotton in many countries since their introduction in 1996 (Bates *et al*., 2005). In India, *Bt* cotton, the only transgenic crop approved so far, was grown on about 5,30,800 ha in the Central and Southern cotton growing regions in 2004 and about 12,00,000 ha in all the three cotton growing regions in 2005. The American bollworm, *Helicoverpa armigera*, a major pest of cotton, that has developed resistance to many insecticides in response to their extensive use is susceptible to Cry1Ac toxin in the *Bt* transgenic cotton. However, like insecticide resistance, it is feared that *H. armigera* might also develop resistance to Cry1Ac toxin in *Bt* cotton, as the selection pressure of *Bt* cotton increases over the years. We have extensively reported up on spatial baseline susceptibility of *H. armigera* populations collected from many locations in the country to *Bt* var. *kurstaki* and its toxins (Gujar *et al*., 2000; 2004: Chandrashekar *et al*., 2005). Similar studies on spatial variation in *H. armigera* susceptibility have been reported in the past (Kranthi *et al*., 2001; Jalali *et al*., 2004).

These studies point out to the presence of natural variation in insect susceptibility; since most were prior to the introduction of *Bt* cotton in 2002. It is only in 2004 that *Bt* cotton cultivation recorded a significant increase (6-fold) over the previous year. Cumulatively, 18,46,430 ha were cultivated under *Bt* cotton in the last four years (2002-2005). Besides, there is illegal *Bt* cotton of the same area or may be more than the legal *Bt* cotton (Jayaraman, 2004). It is therefore in this context, we studied response of *H. armigera* to *Bt* Cry1Ac and as a part of regulatory requirement with the following specific objective: Resistance monitoring of cotton bollworm, *H. armigera* collected from 20 different trial locations to Cry1Ac toxin from JK hybrid cotton.

MATERIALS AND METHODS

Collection and maintenance of H. armigera populations:

H. armigera were collected as larvae from host crops especially cotton from the sites of trials during the course of study. The larvae were fed until pupation in the laboratory on the chickpea-based meridic diet of Nagarkatti and Prakaash adapted by Gujar *et al.* (2004). The dry matter content of chickpea-based meridic diet was ca. 27 g, which included chickpea flour (21 g), yeast (2.75 g), casein (1.25 g), ascorbic acid (0.75 g), methyl *p*-hydroxy benzoate (0.5 g), sorbic acid (0.25 g), streptomycin (0.05 g), cholesterol (0.05 g), multivitamin forte drops (Pfizer®) (50 µl), tocoferol (0.025 g) and formaldehyde (0.25 ml) in 100 ml of water. This was blended with hot homogenate of agar-agar (2.75 g) boiled in 56.25 ml water, allowed to cool and form a cake in the glass Petri-plates at the room temperature. The adults emerging from pupae were offered 10% honey solution fortified with multivitamins throughout their egg-laying period. Five

pairs of adults were kept in each jar covered with rough cotton cloth for egg laying. The neonates on egg hatching belonged to F_1 generation and were used in bioassays to determine baseline susceptibility of *H. armigera*.

Preparation of Cry1Ac toxin:

Cry1Ac toxin was prepared from cotton seed powder of transgenic JK hybrids. It was quantified using ELISA kit of EnviroLogix, USA and made available to us for bioassays. Besides, another set of toxin was prepared by using recombinant *Escherichia coli* JM 103 strain containing hyper-expressing recombinant plasmid vectors pKK223-3 (BGSC ECE53) obtained from Bacillus Genetic Stock Center, Ohio State University, Columbus, USA. It was cultured in nutrient broth at 37°C for 48 h. Toxins were purified as per Lee *et al.* adapted by Gujar et al. (2004). The final pellet was solubilized (50 mM sodium carbonate, pH 10.5, 10 mM dithiothreitol) and centrifuged at 9168 x g for 10 min, and the supernatant containing the solubilized protoxin (>80% pure) was treated with trypsin (10:1 v/v). The toxin obtained was quantified by elution of Coomassie brilliant blue (CBB) R-250 from stained bands after SDS-PAGE using BSA for the standard curve or EnviroLogix's ELISA kit.

Bioassays

Bioassays were carried out by the diet incorporation method as per Gujar et al. (2000). A series of concentrations of Cry1Ac were prepared and added to an aliquot of diet, mixed thoroughly, and transferred to small plastic containers. Each container served as one replicate; with three to five replications per concentration. Ten neonates were released on treated diet (3g diet block) per replication. The control consisted of normal meridic diet (without toxin). A minimum of 210 neonates was used for each bioassay. The mortality

was then pooled for each concentration. The concentrations showing corrected mortality between 20 and 80% at 96 h and 7 days were used for calculation of median lethal concentration (LC_{50}). The experiments with larval mortality of above 10% in control were discarded and repeated. All bioassays were carried out at 27±2 °C and 60-80% r.h. Bioassays were also carried out using terminal cotton leaves of JK Bt cotton hybrids collected from the trial sites and supplied by the company. Each leaf was fed to a group of 10 neonates, with three replications. The control leaves were that of non-Bt cotton hybrid. The mortality was observed at 96 hr. to assess the effectiveness of Cry1Ac toxin. The cotton seeds of Bt hybrids were finely grounded and mixed with artificial diet at the three different concentrations 0.33, 3.33 and 33.3%. Similar controls were kept using non-Bt seeds for all the Bt hybrids used in the study. The neonates, 10 in number, were released on each replicate (of 3 gm diet). Three replicates were used for each treatment (concentration). The mortality was observed at 96 h. The % mortality was corrected using the control. The corrected percent mortality was subjected to analysis of variance.

Comparative studies on the toxicity of Cry1Ac from two different sources viz., JK cotton hybrid seeds and *E. coli* strain (BGSC ECE53) were carried out on the neonates of *H. armigera* collected from IARI farm which is often used as reference strain. Observations on mortality were recorded every 24 h till 96 h. The treatment mortality was corrected for control mortality using Abbott's formula. The LC_{50} were estimated as given below.

Statistical analysis

The mortality data were analyzed by using maximum likelihood program for probit analysis. LC_{50} was expressed in terms of endotoxin concentration (µg gm^{-1} diet). The analysis of variance for the data on mortality with respect to cotton leaves, or seed

powder-concentration combination was carried out with the Duncan's Multiple Range Test (DMRT) at 5% level of probability using Genstat software (Indostat Services, Hyderabad).

RESULTS

Susceptibility of *H. armigera* to *Bt* Cry1Ac *in situ* plant:

Studies were carried out to test the toxicity of terminal leaves of Bt cotton hybrids viz., JK Durga, JK Ishwar, JK Varun, JKCH 226 and JKCH 1947 vis-à-vis their non-*Bt* counterparts as control. The mortality data on 96 h of feeding, after correction for control, was subjected to analysis of variance and it was found that all Bt hybrids were equally effective against the neonates of *H. armigera* (Fig.1).

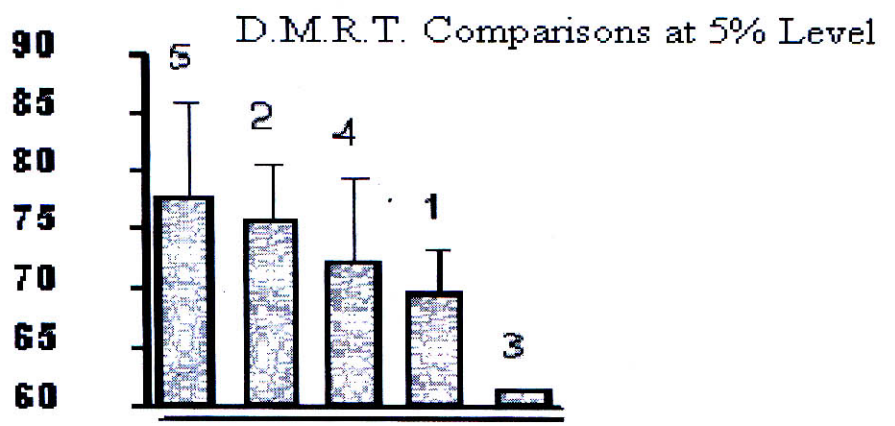

Figure 1. Toxicity of leaves of *Bt* hybrids to neonates of *H. armigera* (% corrected mortality of 1. JK Durga, 2. JK Ishwar, 3. JK Varun, 4. JKCH 226 and 5. JKCH 1947). Toxicity of all leaves to neonates was at par as per DMRT test

Besides, toxicity of seed powders of JK cotton hybrids at three different concentrations each viz., 0.33, 3.33 and 33.3% was studied to neonates of *H. armigera* (Fig. 2). It was observed that Cry1Ac *Bt* seed powders were highly toxic to neonates and toxicity was

dose-dependent. The seed powders of *Bt* hybrids viz., JK Ishwar and JKCH 1947 were highly toxic and at par; followed by JK Varun and then a group of JKCH 226 and JK Durga.

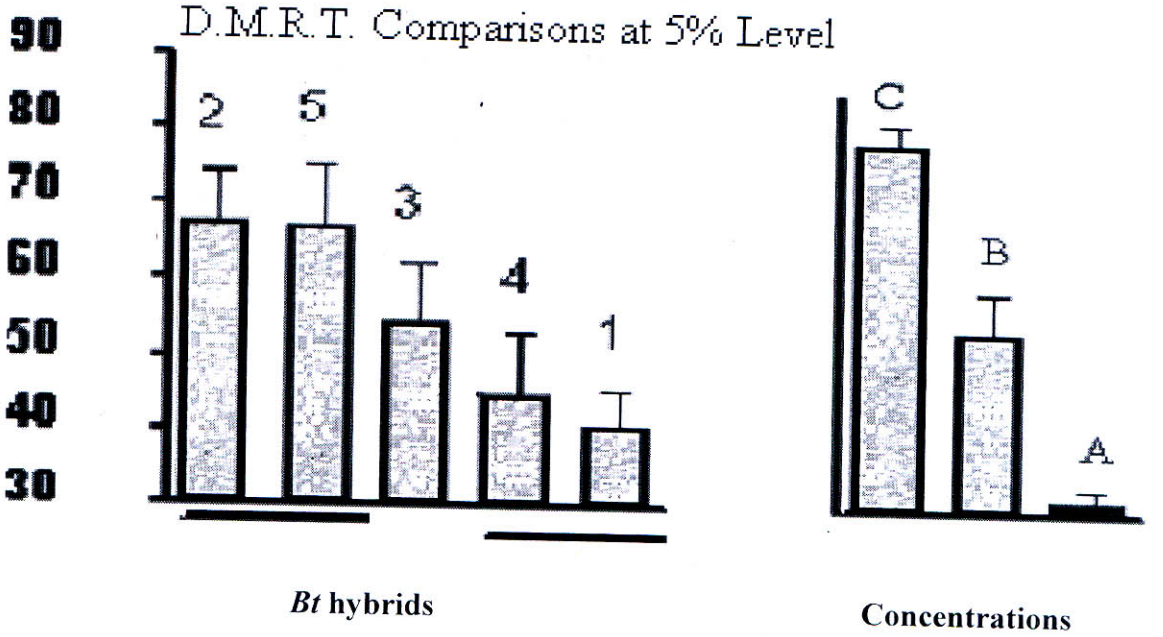

Figure 2. Toxicity of seed powders of *Bt* hybrids to neonates of *H. armigera* on the basis of all concentrations (% corrected mortality of 1. JK Durga, 2. JK Ishwar, 3. JK Varun, 4. JKCH 226 and 5. JKCH 1947 at three different concentrations viz. A. 0.33, B. 3.33 and C. 33.3%

Comparative toxicity of Cry1Ac from two different sources:

Studies were carried out to test the toxicity of Cry1Ac from two different sources as mentioned above to the neonates of *H. armigera* of IARI which is used as reference strain (Table 1). The LC_{50} of Cry1Ac extracted from JK hybrid seed was 1.98 µg/gm diet while that from *E. coli* clone was 4.50 µg/gm diet. Although toxicities of Cry1Ac from two different sources did not differ significantly; nevertheless, Cry1Ac extracted from JK

cotton hybrids were 2.3 times more toxic than that from *E.coli* clone, which could be attributed to the fact that Cry1Ac in JK cotton hybrid was about 60 kDa proteolytically stable toxin that does not undergo any further proteolysis and effectively act on the midgut of target insect species.

Table 1. Comparative toxicity of Cry1Ac from two different sources to neonates of *H. armigera* of IARI used as reference strain

Cry1Ac Source	LC_{50} (96 h) µg/gm	95% Fiducial limits		Slope ±s.e.	Ratio
		Lower	Higher		
Extracted from JK Hybrid cotton seeds	1.98	1.35	2.94	1.74±0.38	1.0
Extracted from *E. coli* (BGSC ECE53)	4.50	2.85	8.74	1.01±0.19	2.3

Baseline susceptibility of 20 different populations to Cry1Ac extracted from JK cotton hybrids:

In 2005; larvae of *H. armigera* were collected from locations viz., Hisar, Sirsa, Karnal, Faridkot, Panchkula, Ludhiana, Akola, Jalna, Rahuri, Nanded, Nagpur, Indore, Khandwa, Katni, Satna, Vadodara, Hyderabad, Basurgaddy, Raichur, and Coimbatore; with New Delhi (IARI) population as reference. The baseline susceptibility (LC_{50}) of neonates belonging to F_1 generation ranged from 0.20 µg/gm diet (the most susceptible Rahuri population) to 5.76 µg/gm diet (the most tolerant Nanded population). The range of insect susceptibility was about 29-fold (Table 1) on the basis of 4-day bioassay period (Table 2). Studies carried out using 7-day bioassay period showed range of LC_{50} from 0.02 µg/gm diet (the most susceptible Rahuri population) to 1.12 µg/gm diet (the most tolerant Nanded population). Thus, the insect susceptibility ranged about 56-fold on the basis of 7-day bioassays (Table 3).

Table 2. Susceptibility of neonates of *H. armigera* populations to Cry1Ac extracted from JK cotton seed on the basis of 4-day bioassays

S. No.	Place of insect collection	Date of Collection	LC_{50} µg/gm of diet (FL at 95%)	LC_{90}µg/gm of diet (FL at 95%)	Slope ± S.E	Resistance Ratio
1.	Akola	Jan. 29, 2005	3.36 (2.17-9.55)	16.42 (6.77-199.86)	1.9±0.5	16.8
2.	Indore	Nov. 6, 2004	2.19 (1.70-2.93)	7.9 (5.06-20.33)	2.3±0.5	10.9
3.	Coimbatore	Feb. 24, 2005	0.83 (0.66-1.09)	3.33 (2.20-6.91)	2.1±0.3	4.15
4.	Nanded	Oct.10, 2004	5.76 (3.16-44.73)	86.84 (18.70-42857)	1.1±0.3	28.8
5.	Satna	Mar. 7, 2005	0.55 (0.36-0.79)	4.03 (2.47-8.87)	1.5±0.2	2.8
6.	Rahuri	Mar. 9, 2005	0.20 (0.10-0.31)	1.71 (1.05-3.77)	1.4±0.2	1
7.	IARI (New Delhi)	Mar.17, 2005	1.98 (1.35-2.94)	10.76 (5.95-41.42)	1.7±0.4	9.9
8.	Ludhiana	Mar.25, 2005	0.41 (0.22-0.53)	5.76 (3.00-19.05)	1.1±0.2	2.1
9.	Hisar	Apr. 1, 2005	0.37 (0.26-0.51)	1.61 (1.11-2.82)	2.0±0.3	1.8
10.	Karnal	Apr. 9, 2005	0.33 (0.21-0.47)	1.96 (1.29-3.75)	1.7±0.2	1.6
11.	Sirsa	Apr. 13, 2005	1.37 (0.96-2.0)	9.7 (5.48-26.06)	1.5±0.2	6.8
12.	Faridkot	Apr. 24, 2005	0.20 (0.12-0.28)	0.79 (0.54-1.43)	2.1±0.4	1
13.	Punchkula	Apr. 22, 2005	0.64 (0.28-1.18)	23.02 (7.47-393.06)	0.8±0.2	3.2
14.	Vadodara	Oct. 7, 2005	0.95 (0.63-1.39)	8.13 (4.50-22.51)	1.4±0.2	4.7
15.	Hyderabad	Sep. 2, 2005	0.52 (0.31-0.79)	4.87 92.76-13.34)	1.3±0.2	2.6
16.	Basurgaddy (RR distt)	Sep. 2, 2005	0.35 (0.20-0.55)	4.31 (2.38-11.2)	1.2±0.2	1.7
17.	Katni	Sep. 1, 2005	0.54 (0.34-0.83)	6.26 (3.44-16.38)	1.2±0.17	2.7
18.	Jalna	Oct. 27, 2005	1.04 (0.70-1.7)	9.68 (4.61-37.73)	1.3±0.2	5.2
19.	Khandwa	Oct. 10, 2005	0.36 (0.21-0.58)	5.88 (2.68-26.59)	1.1±0.2	1.8
20.	Raichur	Oct. 7, 2005	1.62 (1.08-2.96)	14.12 (6.26-69.49)	1.4±0.2	8.1
21.	Hisar	Oct. 21, 2005	0.92 (0.49-2.39)	44.02 (9.72-2439.31)	0.8±0.2	4.6
22.	Sirsa	Oct. 19, 2005	1.08 (0.76-1.64)	7.13 (3.88-20.86)	1.6±0.2	5.4
23.	Nagpur	Nov. 11, 2005	1.00 (0.67-1.57)	7.05 (3.68-25.21)	1.5±0.3	5.0

Hisar and Sirsa populations were repeated in view of their availability and IARI (New Delhi) was used as a reference

Table 3. Susceptibility of neonates of *H. armigera* populations to Cry1Ac extracted from JK cotton seeds on the basis of 7-day bioassays

S. No.	Place of insect collection	Date of Collection	LC_{50} µg/gm of diet (FL at 95%)	LC_{90}µg/gm of diet (FL at 95%)	Slope ± S.E	Resistance Ratio
1.	Akola	Jan. 29, 2005	0.76 (0.59-0.98)	2.52 (1.81-4.25)	2.5±0.3	38.0
2.	Indore	Nov. 6, 2004	0.79 (0.56-1.02)	2.93 (2.13-5.06)	2.3±0.4	39.5
3.	Coimbatore	Feb. 27, 2005	0.11 (0.03-0.19)	0.67 (0.46-1.27)	1.7±0.4	5.5
4.	Nanded	Oct.10, 2004	1.12 (0.80-1.46)	5.46 (3.56-12.25)	1.9±0.3	56.0
5.	Satna	Mar. 7, 2005	0.094 (0.03-0.2)	2.58 (1.18-65.6)	1.5±0.3	4.7
6.	Rahuri	Mar. 9, 2005	0.02 (0.0-0.05)	0.14 (0.02-0.35)	1.1±0.6	1
7.	IARI (New Delhi)	Mar.17, 2005	0.37 (0.22-0.53)	1.77 (1.15-3.43)	1.9±0.3	18.5
8.	Ludhiana	Mar.25, 2005	0.18 (0.1-0.26)	0.92 (0.61-1.76)	1.8±0.3	9
9.	Hisar	Apr. 1, 2005	0.10 (0.04-0.17)	0.61 (0.39-1.22)	1.7±0.3	5.0
10.	Karnal	Apr. 9, 2005	0.25 (0.17-0.35)	1.14 (0.78-2.06)	1.9±0.3	12.5
11.	Sirsa	Apr. 13, 2005	0.26 (0.12-0.42)	3.34 (1.85-9.58)	1.2±0.2	13.0
12.	Faridkot	Apr. 24, 2005	0.07 (0.02-0.11)	0.26 (0.17-0.63)	2.2±0.6	3.5
13.	Punchkula	Apr. 22, 2005	0.12 (0.04-0.22)	1.30 (0.76-3.25)	1.2±0.3	6.0
14.	Vadodara	Oct. 7, 2005	0.21 (0.09-0.34)	2.51 (1.43-6.76)	1.2±0.2	10.5
15.	Hyderabad	Sep. 2, 2005	0.15 (0.07-0.25)	1.17 (0.72-2.56)	1.4±0.3	7.5
16.	Basurgaddy (RR dist)	Sep. 2, 2005	0.096 (0.05-0.15)	0.74 (0.45-1.61)	1.4±0.2	4.8
17.	Katni	Sep. 1, 2005	0.18 (0.11-0.26)	1.13 (0.72-2.22)	1.6±0.2	9.0
18.	Jalna	Oct. 27, 2005	0.24 (0.15-0.36)	2.37 (1.35-6.15)	1.3±0.2	12
19.	Khandwa	Oct. 10, 2005	0.15 (0.07-0.24)	1.82 (1.00-5.20)	1.2±0.2	7.5
20.	Raichur	Oct. 7, 2005	0.47 (0.29-0.76)	6.79 (3.11-29.58)	1.1±0.2	23.5
21.	Hisar	Oct. 21, 2005	0.08 (0.04-0.133)	0.81 (0.48-1.99)	1.3±0.2	4.0
22.	Sirsa	Oct. 19, 2005	0.26 (0.17-0.37)	1.86 (1.14-4.00)	1.5±0.2	13.0
23.	Nagpur	Nov. 11, 2005	0.25 (0.17-0.34)	0.95 (0.65-1.62)	2.2±0.3	13.0

Hisar and Sirsa populations were repeated in view of their availability and IARI (New Delhi) was used as a reference

DISCUSSION AND CONCLUSIONS

Review of literature on the efficacy of Cry1Ac either as a toxin in artificial diet or *in situ* in *Bt* transgenic leaves showed that Cry1Ac is the most toxic of all the toxins to neonates of *H. armigera* (Sachs *et al.*, 1998; Gujar *et al.*, 2000; Adamczyk *et al.*, 2001; Kranthi *et al.*, 2001; Fakrudin *et al.*, 2003; Chandrashekar *et al.*, 2005). Studies carried out by us (Chandrashekar *et al.*, 2005) showed that insect populations from 9 locations in India showed range of insect susceptibility to *B. thuringiensis* var. *kurstaki* strains and individual Cry toxins viz., Cry1Aa 10.5, Cry1Ab 12.8, Cry1Ac 16.2, HD-1 14.1 and HD-73 5.7-fold which was attributed to genetic variability.

Studies on baseline susceptibility of *H. armigera* to Cry1Ac using discriminating dose bioassays were carried out by us (Gujar *et al.*, 2000) for insect populations collected from several cotton growing locations in the country. Later, Kranthi *et al.* (2001) showed 67-fold variation in susceptibility of neonates of *H. armigera* (LC_{50} 0.01-0.67 µg/ml diet 5-day assay). Chandrashekar *et al.* (2005) reported 16-fold variation in susceptibility of neonates of *H. armigera* (LC_{50} 0.023 µg-0.372 µg/ml diet 4-day assay). The effect of host-plant interaction on insect susceptibility was also studied by us (Gujar *et al.*, 2004) which reported that *H. armigera* reared on proteinaceous host crops were more tolerant to Cry1Ac than those on the non-proteinaceous host crops.

Extensive investigations on baseline susceptibility of *H. armigera* to *B. thuringiensis* were also carried out in China. Wu *et al.* (1999) reported about 100-fold variation in susceptibility of *H. armigera* from five ecological cotton areas in China to Cry1Ac.

Further studies on monitoring of insect susceptibility to Cry1Ac were carried out for the populations sampled mostly from *B. thuringiensis* cotton fields by Wu *et al.* (2002). They have found, at the most, five-fold variation in IC_{50} (concentration producing 50% inhibition of larval development to third instar). The IC_{50} ranged from 0.020-0.105 µg/ml, 0.016-0.099 µg/ml and 0.016-0.080 µg/ml for 1998, 1999 and 2000 insect populations, respectively. This is quite interesting in view of the fact that, till todate, there is no evidence of development of field-level resistance to *H. armigera* to Cry1Ac; despite large-scale growing of *Bt* cotton in China wherein no *Bt* resistance management tactics are mandatory for the farmers.

The present investigations clearly showed that the baseline susceptibility data generated herein are in concurrent with that reported in the literature and there is no evidence of resistance development in *H. armigera* over the period of last one year under study. This is inspite of the fact that *Bt* cotton area has increased from 5,30,800 ha in 2004 to about 12,00,000 ha in 2005. It is possible that *H. armigera* has not extensively segregated in to resistant homozygous population in view of its phytophagous nature, high mobility, semi-recessive nature of Bt resistance and the present mandatory *Bt* resistance management tactics being implemented in the country.

Publications/Presentations/Seminars wherein funding received from JK AgriGenetics Ltd is acknowledged

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ACKNOWLEDGEMENTS

We are grateful to Head, Division of Entomology for allowing us to implement the project, to Jt Director (Res) and Director, Indian Agricultural Research Institute, New Delhi for their approval and encouragement; and JK AgriGenetics Ltd. Hyderabad for sponsoring and funding the project. We are especially thankful to guidance and cooperation of Dr. P.S. Bhattacharya, General Manager (Biotech) and Dr. P.S. Dravid, President of JK AgriGenetics Ltd., Hyderabad during the course of implementation of the project.

Summary of the findings of the project entitled

"Baseline susceptibility of *Helicoverpa armigera* to *Bt* Cry1Ac toxin in as many as twenty selected different locations in India for monitoring resistance" (IARI 79-11)

sponsored by

JK AgriGenetics Ltd., Hyderabad

and executed at

Division of Entomology, Indian Agricultural Research Institute, New Delhi

Investigations on the baseline susceptibility of the American bollworm, *Helicoverpa armigera* to Cry1Ac toxin were carried out with an aim to detect and monitor development of resistance, in view of increasing area under Bt cotton hybrids in India in the past four years and as a part of regulatory requirement for the Sponsors. Studies on the susceptibility of neonates of *H. armigera* showed the high toxicity of Cry1Ac *in situ* in the leaves and seeds of JK *Bt* cotton hybrids. Using Cry1Ac from two different sources viz., JK *Bt* cotton and *Escherichia coli* expressing *cry1Ac* gene; toxicity to neonates did not differ significantly. Further, the baseline susceptibility of *H. armigera* to Cry1Ac obtained from JK cotton seeds showed a range of LC_{50} ranging from 0.20 µg/gm (for the most susceptible population, Rahuri) to 5.76 µg/gm (for the most tolerant population, Nanded) and was about 29-fold on the basis of 96h LC_{50} bioassays. It ranged from 0.02 to 1.12 µg/gm and of about 56-fold on the basis of 7-day LC_{50} bioassays. The baseline susceptibility of *H. armigera* to Cry1Ac was found to be high due to genetic diversity of insect populations and does not provide any evidence of field-level resistance development in *H. armigera* during the year under study.

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