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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 cotton incorporating *cry1Ac* gene derived from Bt (*Bacillus thuringiensis*) is one of the most exciting advances made in cotton pest management in recent times. The cotton bollworm *Helicoverpa armigera* (Hübner) is one of the main target pests of Bt cotton technology in India. It has a history of developing resistance to almost all the insecticides used for its control. Cry1Ac is the most toxic of the *Bacillus thuringiensis* insecticidal proteins to *H. armigera*. Laboratory experiments to select for resistance in India, Australia and China have shown that *H. armigera* is capable of developing resistance to Cry1Ac. Bt cotton expresses the Cry1Ac toxin in all parts of the plant throughout the growth period. The pest would thus be exposed to a continuous selection pressure, thereby causing resistance in field populations. It is important to ensure that the technology, which represents the state of art of pest management, remains effective in controlling target pests for the longest possible time. Information on resistance monitoring helps immensely in devising proactive resistance management strategies that can retard the rate of resistance development. 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 2003-2004 cropping season, which is the second 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

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. The *H. armigera* strains were bioassayed in the laboratory on a wheatgerm based semi-synthetic diet. The following diet recipe was used for the bioassays. 160 g chickpea seed-flour, 60 g wheatgerm, 3.3 g methyl parabenzoate, 1.7 g sorbic acid, 5.3 g ascorbic acid, 2.5 g Aureomycin, 16 g agar, 53 g dried active yeast and 1200 ml water. The MVP-II toxins were diluted in water in five concentrations and 5 ml was added to 45 ml diet when the temperature of the diet reached 58⁰C and before it polymerized. The Cry1Ac in the assays ranged from 0.001 - 1.0 µg/ml diet in the bioassays. The mixture was vortexed thoroughly and poured into 25-well insect rearing trays (Innovative Biosciences, Nagpur) approximately at 2ml per well. The diet was cooled and first instar larvae (2-d old) were released at one per well. The plates were incubated at 25⁰C at 70% R.H. The assays were done with at least five concentrations of the toxin and with 2-3 replicates. A total of 20-24 larvae were used per concentration. The larvae were transferred on to fresh toxin-incorporated diet after 3-4 days and incubated until they were 7 days old. Mortality observations and weight of surviving larvae were recorded on the 7th day. Log linear regression analysis was used to determine median lethal concentration (LC₅₀) and median growth inhibition concentration (IC₅₀), and their 95% fiducial limits (FL) were computed by probit analysis. The median growth inhibition concentration (IC₅₀) was derived based on the concentration of Cry1Ac in the diet that inhibited 50% of the test insects from reaching third instar stage. The major advantage with IC₅₀ method is that it is based on

the observation of numbers of third instar larvae not molting to third instar in each of the concentrations and not on weights of individual larvae as is done for the EC (effective growth inhibition concentration) calculations. The results of IC₅₀ and EC₅₀ are almost similar because the weight of larvae on control diet normally reaches 80 mg and the third instars weigh 30-40 mg, which is about 50% of the weight of control larvae.

Sampling regions and field strains

H. armigera larvae were collected from all over the cotton growing regions of the country during the cropping season of 2003. Efforts were made to collect insects from the regions where Bt-cotton was being cultivated, but care was taken to ensure that the larvae were collected from only non-Bt-cotton fields. The strains collected from North India did not survive due to an unknown disease and hence all the cultures were destroyed as a quarantine measure. The laboratory strains of *H. armigera* from Central and South India were established from the field-collected larvae. The larvae were collected from non-Bt cotton fields during September –October 2003, from 12 districts of central India (Nagpur, Akola, Yeotmal, Jalgaon, Surendranagar, Amreli, Bharuch, Rajkot, Vadodara, Surat, Sabarkanta and Junagarh), and during November 2003 from 7 districts of South India (Warangal, Khammam, Karimnagar, Medak, Nalgonda, Mahbubnagar and Rangareddy) 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 27⁰C±1⁰C and 70% R.H.

Results & Discussion

The susceptibility of *H. armigera* to Cry1Ac does not appear to have undergone any significant changes over the previous two years of resistance monitoring. The log dose probit assays showed a consistent conformity to the baseline toxicity values

established in India. The LC₅₀ values ranged from 0.04 to 0.38 µg Cry1Ac/mL of diet to indicate a 10-fold variability in the field strains across the country. There were no distinct differences in the susceptibility of *H. armigera* from South India as compared to the Central Indian populations, an observation that has been generally observed previously. The IC₅₀ values ranged from 0.005 to 0.023 Cry1Ac µg / ml diet, and were within the range of values (0.003 – 0.043 µg Cry1Ac/mL of diet) reported during the previous year. The results thus show clearly that there have been no changes as yet in the overall susceptibility of *H. armigera* to Cry1Ac. Specific efforts were made to collect larger samples from the key cotton growing districts of Gujarat, especially from regions where Bt-cotton was being extensively cultivated. The bioassays showed that all the strains collected from Gujarat were found to be well within the baseline susceptibility range thus far.

The LC₅₀ and IC₅₀ values of Cry1Ac, reported in this report are similar to those reported previously for *H. armigera* strains from India, Australia and China. However, the baseline LC₅₀ susceptibility values of *H. armigera* to Cry1Ac in China (Wu et al., 1999), were found to be very variable with a range from 0.091 to 9.073 µg/ml diet. The baseline LC₅₀ values of 0.01 to 0.67 µg/ml reported by us (Kranthi et al., 2001) previously, and 0.11 to 0.71 µg/ml reported recently by Jalali, et al. (2004), for Indian strains indicate that the Chinese *H. armigera* strains are inherently more tolerant to Cry1Ac than the Indian strains. The baseline range of EC₅₀ values at 0.003 to 0.008 and EC₉₀ 0.009 to 0.076 µg/ml diet, published by Jalali et al. (2004), our previous EC₅₀ data of 0.014 and EC₉₀, 0.084 µg/ml diet, and the current values of the Bt-seed based bioassays at IC₅₀, 0.012 to 0.013 and IC₉₀, 0.091 to 0.109 µg/ml diet, showed that the results of the bioassays on Indian *H. armigera* population were comparable even when performed independently in laboratories across the country.

Baseline studies provide a benchmark for the susceptible response of insect species. However, detection of resistant genotypes and monitoring the increase in their frequencies is crucial for resistance management. Dose regression assays indicate shifts in the baseline, but may not assist in detecting resistant genotypes or even indicate the onset of resistance. Discriminating dose assays clearly define the toxic level of the insecticide that can detect resistant genotypes. Thus a single dose would then enable monitoring of an increase in the frequencies of resistant alleles in field populations, as influenced by selection pressure. However, discriminating dose assays for mortality were found to be unreliable in differentiating resistant and susceptible populations of *Heliothis virescens* and *Helicoverpa zea*. Therefore the correct discriminating assay was designated to assess growth-regulating effects based on the capability of a single dose (IC₉₉) to prevent at least 99% larvae from reaching the third instar stage in susceptible populations. Thus the discriminating dose would be capable of detecting heterozygous individuals having single resistant allele with partial dominance. Wu et al., (2002) monitored resistance using the discriminating dose method and showed that IC₉₉ was a preferred indicator of resistance development. The current study shows that if the highest dose in this study of 1.0 µg/ml was considered as a diagnostic dose (similar to the one used in China), then it is clear that there were no third instar larvae that survived this dose.

For sustainable efficacy of Bt-cotton transgenic crops, it is necessary that the susceptibility of *H. armigera* does not undergo any major shifts towards tolerance or resistance. 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. 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. 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 changes that would have occurred in Cry1Ac susceptibility among *H. armigera* populations from different geographic locations within India, after the introduction of Bt-cotton. 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; Jalali *et al.*, 2004) and the related species *H. virescens* and *H. zea* (Sims *et al.*, 1996). The variability in toxicity during 2003 was to an extent 10 fold to Cry1Ac. Compared to our (Kranthi *et al.*, 2001) earlier estimate of 67 fold, and the previous years variability of 32 fold, the current value seems to indicate a decreased variability in response of *H. armigera* to Cry1Ac. The current data show that two years 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 probable that the intense dispersal and migration of the species is one of the reasons for a continuous dilution of resistant allele frequencies in regions where Cry1Ac selection pressure is operational in the form of Bt-cotton. Also adoption of Bt cotton is in its initial phase in India and has not yet reached even > 5% of the cotton area. Hence, the continuation of *H. armigera* susceptibility to Cry1Ac confirming to the established baseline values, is not surprising.

Table 1. Baseline susceptibility: Lethal concentration (LC₅₀) of Cry1Ac to the cotton bollworm *Helicoverpa armigera*. Data of strains collected from nineteen cotton growing districts of India.

Strain	Date	n	LC₅₀	95% F.L	LC₉₀	Slope ± SE
Rangareddy	Nov-03	120	0.259	0.09 - 1.26	3.85	1.09 ± 0.2
Khammam	Nov-03	100	0.38	0.21 - 0.96	6.23	1.06 ± 0.2
Mahbubnagar	Nov-03	80	0.09	0.05 - 0.17	0.71	1.5 ± 0.3
Nalgonda	Nov-03	100	0.13	0.03 - 0.50	1.29	1.3 ± 0.2
Medak	Nov-03	100	0.15	0.04 - 0.51	1.05	1.5 ± 0.2
Warangal	Nov-03	120	0.24	0.09 - 0.96	3.36	1.12 ± 0.2
Karimnagar	Nov-03	120	0.34	0.14 - 14.0	4.05	1.18 ± 0.2
Nagpur	Sep-03	120	0.03	0.008 - 0.05	0.88	0.86 ± 0.2
Jalgaon	Sep-03	120	0.04	0.01 - 0.09	0.26	1.57 ± 0.3
Akola	Sep-03	120	0.16	0.07 - 0.37	1.17	1.47 ± 0.2
Yavatmal	Sep-03	120	0.17	0.10 - 0.27	1.51	1.34 ± 0.2
Surendranagar	Oct-03	100	0.15	0.06 - 0.40	0.72	1.89 ± 0.3
Amreli	Oct-03	100	0.09	0.06 - 0.14	0.38	2.09 ± 0.3
Bharuch	Oct-03	100	0.15	0.10 - 0.21	0.54	2.27 ± 0.3
Rajkot	Oct-03	100	0.19	0.11 - 0.36	2.29	1.19 ± 0.2
Vadodara	Oct-03	100	0.14	0.09 - 0.20	0.49	2.32 ± 0.4
Surat	Oct-03	120	0.25	0.10 - 0.86	1.86	1.46 ± 0.2
Sabarkanta	Oct-03	120	0.37	0.25 - 0.58	1.99	1.76 ± 0.3

Table 2. Baseline susceptibility: Growth inhibitory concentration (IC₅₀) of the Cry1Ac to cotton bollworm *Helicoverpa armigera*. Data of strains collected from nineteen cotton growing districts of India.

Strain	Date	n	IC₅₀	95% F.L	IC₉₀	Slope ± SE
Rangareddy	Nov-03	120	0.011	0.004 - 0.02	0.07	1.58 ± 0.4
Khammam	Nov-03	100	0.014	0.006 - 0.02	0.07	1.82 ± 0.4
Mahbubnagar	Nov-03	80	0.014	0.004 - 0.02	0.08	1.62 ± 0.4
Nalgonda	Nov-03	100	0.005	0.0004 - 0.01	0.05	1.28 ± 0.4
Medak	Nov-03	100	0.006	0.0009 - 0.01	0.05	1.49 ± 0.4
Warangal	Nov-03	120	0.021	0.005 - 0.04	0.14	1.58 ± 0.3
Karimnagar	Nov-03	120	0.023	0.009 - 0.04	0.11	1.89 ± 0.4
Nagpur	Sep-03	120	0.009	0.002 - 0.02	0.08	1.37 ± 0.3
Jalgaon	Sep-03	120	0.007	0.001 - 0.01	0.09	1.17 ± 0.3
Akola	Sep-03	120	0.012	0.005 - 0.02	0.05	2.01 ± 0.4
Yavatmal	Sep-03	120	0.017	0.009 - 0.02	0.07	2.03 ± 0.4
Surendranagar	Oct-03	100	0.021	0.009 - 0.03	0.12	1.63 ± 0.3
Amreli	Oct-03	100	0.012	0.004 - 0.02	0.06	1.77 ± 0.4
Bharuch	Oct-03	100	0.011	0.003 - 0.02	0.07	1.57 ± 0.4
Rajkot	Oct-03	100	0.014	0.005 - 0.02	0.11	1.46 ± 0.3
Vadodara	Oct-03	100	0.015	0.006 - 0.02	0.08	1.75 ± 0.4
Surat	Oct-03	120	0.016	0.008 - 0.025	0.08	1.85 ± 0.4
Sabarkanta	Oct-03	120	0.018	0.002 - 0.04	0.12	1.54 ± 0.3

PART-II

Baseline toxicity of Cry 1 Ac toxin against spotted bollworm, *Earias vittella* (Fab) using diet based bioassay -2003

The spotted bollworm, *Earias vittella* (Fab.) is one of the bollworms attacking cotton during the early and mid- season of crop growth. In the current year we determined the baseline data on the toxicity of Cry1Ac to *E. vittella* strains collected from eleven cotton growing districts from north and central India. 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.

Larvae were collected from 11 cotton-growing districts of north and central India during September and November 2003. The collections were mainly from 6 districts from central India and 4 districts of north India. The districts from central India were Surendranagar, Amreli, Surat, Sabarkanta, Bhavnagar and Bharuch from Gujarat. In north India the districts were, Hanumangarh and Sriganganagar from Rajasthan, Sirsa from Haryana and Mansa from Punjab. 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 constituents of which are mentioned in Table 3. The diet was poured out in multi-cell 12-well plates and larvae were reared singly until pupation. Subsequent rearing of pupae and adults were similar to the protocol described earlier. To prevent early neonatal mortality, which occurs generally on the wet surface of semi-synthetic diet, neonates were reared on tender

terminal cotton leaves for the first two days before being transferred on to diet, with or without the toxin.

Cry1 Ac toxin protein was produced according to Albert and co-workers (1991) from *E. coli* strains containing hyper expressing recombinant plasmid vector pKK 223-3 kindly provided by Dr.Zeigler, Ohio State University, US. The toxin was purified from over expressing cells by sonication and extensive washing with 10 % sodium bromide. Proteins were quantified according to Lowry's method and the toxin was quantified on SDS-PAGE densitometry before preparing dilutions of six to ten concentrations (ranging from 10 to 20,000 fold) in distilled water. The protein thus produced contained 38 per cent of the full-length Cry1Ac toxin. Because transgenic cotton produces the non-activated, full length Cry1Ac protein (~ 130 KD), the LC₅₀ and LC₉₀ values were determined for the full-length Cry1Ac toxin and expressed as µg Cry1Ac per ml diet.

A new bioassay method was designed keeping in view the low moisture requirement, of *E. vittella* larvae. Cry1Ac was diet incorporated at six concentrations viz. 416, 104, 52, 10.4, 2.6 and 1.3 ng / ml of diet. Diet was cooled to 55⁰ C before addition of toxin. Toxin incorporated diet (10 ml) was poured on to five filter paper strips of 1 X 0.5'' 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. At least 10 larvae were released per concentration with 4-6 concentrations per assay, including controls. All assays were replicated two or three times and pooled data were subjected to analysis. The assay cups were maintained at 27⁰ C ± 1⁰ 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 until the end of the experiment. Controls were maintained on plain diet strips. Concentration-mortality regressions were estimated using POLO PC statistical package.

The diet tested was found to be suitable for insect growth and did not involve the use of seed flour of either cotton or Okra (*Abelmoschus esculentis* Moench), as was reported earlier. Over the six continuous generations of testing, there were no significant differences in any of the growth parameters of the insect cultures. Through the F₁-F₆ generations, hatching was 76 ± 5 %, larval period was 14 ± 2 days and pupal period averaged at 10 ± 1 days. The cost of diet worked out to be \$10 per litre that could support 3000 larvae of *E. vittella* in the younger instars and 720 of the 3-5th instar larvae for at least 3 days. It is recommended that 6-day-old larvae of *E. vittella* be reared singly until pupation since they demonstrate cannibalistic behavior especially under conditions of over crowding or starvation.

The bioassay protocol is simple, as larvae do not require frequent handling, by transfer from one cup to another. Instead, fresh diet strips could be replaced every alternate day in the rearing containers. Spreading of 10 ml of diet over five 1 X 0.5" filter paper strips ensured 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 also the formation of bacterial molds. Change of diet strips every alternate day was 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*.

Cry1Ac was found to be highly toxic to the spotted bollworm *E. vittella*. Baseline toxicity data for 2003 are presented in Table 4. The LC₅₀ of Cry1Ac ranged from 0.01 to 0.028 µg/ml of diet, 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 as compared to the controls, especially in individuals treated with highest toxin dose.

The leaf-dip assay that was used by us (Kranthi et al., 1999) previously to report baseline susceptibility of *E. vittella* to Bt toxins in India, certainly represents toxic responses that are close to the natural conditions, since it facilitates the exposure of the insect to toxins layered on the cotton leaf. But, the bioassay method may suffer from disadvantages of being influenced by extraneous factors that are associated with the leaf such as variety, age of the leaf, stage of the plant etc. apart from the biotic and abiotic factors that have an impact on health of the leaf. The diet based bioassay circumvents these problems and is fairly robust to assess only susceptibility changes in the test insect, neither being influenced by the host plant leaf matrix, nor having to depend on availability of plant parts for the assay. Moreover the diet based assays facilitate replicable resistance monitoring systems in various parts of the country, which would be based on a common diet recipe and a common source of toxin so that the results would be reliable and comparable.

The variability in the strains was less than 10-fold. The bioassay response in the central and north Indian strains was consistent with a minimum variability at 2.3 and 2.7-fold respectively.

Our previous results with Cry1Ac leaf-dip bioassays on *E. vittella* strains collected from central and north India showed that the average LC₅₀ and LC₉₉ values were 0.88 and 627ng/sq cm respectively. When extrapolated to the expression in transgenic plants, an application of 1ng/ cm² in the leaf-dip assay would be equivalent to 0.25µg/gm dry weight of the tissue (The dry weight of a leaf disc of 1 cm² equals 4mg). Thus if Bt cotton expressed Cry1Ac at 0.22 µg/gm dry weight of the tissues, it would equal the median lethal concentration of 0.88ng/sq cm of the leaf tissue on dry weight basis. In the current study the mean LC₅₀ value of 0.024 µg Cry1Ac per ml diet would be equivalent to 0.19µg/gm dry weight of the diet (the wet weight of semi-synthetic diet is ≈ 8.0 times that of the oven dried diet). Hence in terms of Cry1Ac equivalence, and the toxicity response to *E. vittella*, both the leaf-dip assays and diet-based assays are comparable.

Bt cotton is the first transgenic to be introduced in India. Resistance management programs have to be designed for the Indian situation with 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. It is therefore necessary to regularly monitor shifts in baseline susceptibility of *E. vittella* in regions especially where Bt cotton is grown and also to periodically assess the changes in efficacy of Bt cotton over time. It is also pertinent to develop and initiate implementation of proactive resistance management strategies to ensure that the rate of resistance development is retarded.

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Table3: Diet recipe for the spotted bollworm, *Earias vittella*

Ingredients	Quantity per litre of diet (g)
Wheat germ	96
Caesin	44
Sucrose	39
Ascorbic acid	5
Sorbic acid	2
Methyl para hydroxy benzoate	2
Choline chloride	1.25
Cholesterol	1.25
Wessons salt	12.5
Yeast	19
Agar Agar	25
Formal dehyde (5%)	20ml
Becosule syrup	10ml
Water	1100ml

Table 4: Log dose probit response of field populations of *E. vittella* to Cry1Ac.

District	Collection date	n	*LC₅₀	95% F.L	*LC₉₀	Slope ± SE
<u>Central India</u>						
Surendranagar	November 03	182	0.023	0.013-0.039	0.06	3.3 ± 0.6
Amreli	November 03	400	0.010	0.002-0.018	0.07	1.5 ± 0.2
Surat	November 03	407	0.017	0.014-0.021	0.06	2.3 ± 0.3
Sabarkantha	November 03	250	0.017	0.008-0.027	0.13	1.4 ± 0.2
Bhavnagar	November 03	114	0.023	0.007-0.050	0.11	1.8 ± 0.3
<u>Bharuch</u>	November 03	233	0.023	0.014-0.070	0.21	1.6 ± 0.2
<u>North India</u>						
Hanumangarh	September 03	230	0.017	0.006-0.030	0.09	1.8 ± 0.3
Sriganganagar	September 03	210	0.013	0.008-0.018	0.05	2.3 ± 0.6
Sirsa	September 03	150	0.028	0.010-0.063	0.14	1.9 ± 0.3
Mansa	September 03	150	0.011	0.004-0.017	0.05	2.1 ± 0.5

*LC₅₀, LC₉₀ values are expressed as µg Cry1Ac per ml of diet

n refers to the total number of insects tested in the two-three replicate bioassays.

F. L refers to fiducial limits.

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