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Changes in baseline susceptibility of the cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) to Cry1Ac toxin from *Bacillus thuringiensis.*

Abstract

Changes in the geographical variability in *H. armigera* susceptibility levels to Cry1Ac toxin from *Bacillus thuringiensis* were monitored through log dose probit assays conducted on populations collected from 10 cotton-growing districts of North India, 26 districts of Central India and 13 districts of South India. The LC_{50} values derived from 17,330 larvae tested, ranged from 0.057 to 1.146 µg Cry1Ac/ml of diet with 8.5-fold, 16.61- fold and 14.88 –fold variability in susceptibility across the North, Central and South Indian strains of *H. armigera*. The IC_{50} values ranged from 0.009- 0.201 µg Cry 1Ac/ml of diet with 22.33 fold variability across the country.

Introduction

Insecticidal genes belonging to the Cry1A group have been the most widely used for the generation of transgenic crops. Cry1Ac 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. Bollgard, which expresses a single gene *cry1Ac* has been in cultivation in the USA for almost a decade and is considered as one of the best technological advances for cotton pest management. Bt cotton was introduced in India since 2002 and over six years its area has increased from 30,000 hectares to 63 lakh hectares in 2007-08 with no report of field control failures of *H. armigera*, so far. A total of 135 Bt hybrids are currently available in the Indian market. Choice of the transgenic cotton genotype available to the Indian farmer is the highest amongst transgenic cotton growing countries in the world while the events are just four. However, the deployment of a single gene for the expression of insecticidal toxin protein in crop plants is expected to select for resistance in the target pests over a period of continuous exposure. Rapid responses to laboratory selection, show that many pests naturally harbor genetic variation in susceptibility to Bt toxins and thus have the potential to evolve resistance to Bt crops in the field [2-5]. The capacity of *H. armigera* to develop resistance to Cry1Ac has been demonstrated by laboratory selection in Australia[1], China[6] and India[7]. Analysis of more than a decade of global monitoring data reveals that the frequency of resistance alleles has increased substantially in some field populations of *H. zea* but not in *H. armigera, Heliothis virescens* or *Pectinophora gossypiella*. The resistance of *H. zea* to Cry1Ac in transgenic cotton has not caused widespread crop failures, in part because other tactics augment control of this pest [8.] Although field control failures of bollworms have not yet been reported from any country including India, till date, availability of full grown *H. armigera* larvae on Bt cotton especially in Gujarat is gradually increasing over the years. However, populations of *H. armigera* collected from Bt cotton are not being used to monitor changes in baseline susceptibility. Populations are specifically collected from non-Bt cotton if available or on hosts other than cotton.

Widespread and prolonged exposure to Bt toxins represents one of the largest selections for resistance in insect populations the world has ever seen [9]. In order to delay the development of resistance, resistance management programs need to be in place. 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[10]. Since the first studies wherein baseline monitoring for the bollworms was reported [11-14] CICR has developed a database on the changes in baseline susceptibility to Cry1Ac in *H. armigera* monitored each year from populations collected on cotton and on other crops. It is important that resistance development to the Cry1Ac in the target pest is monitored carefully so that timely management measures can be initiated. The current study aims to understand the changes in geographical variability of the baseline susceptibility in the cotton bollworm, *H. armigera* to the Cry1Ac toxin in North, Central and South India six years after introduction of Bollgard for commercial cultivation.

Materials and Methods

Laboratory strains of *H. armigera* were established from larvae collected in cotton fields during the cropping season of 2007-2008 from major cotton growing regions of North India. Field strains of the cotton bollworm *H. armigera* were collected during December 2007, on chickpea from 10 districts of three states in North India (Sirsa and Hissar, Hanumangarh of Haryana, Mansa, Bhatinda, Fatehabad, Abohar, Ludhiana of Punjab and Sriganganagar of Rajasthan) and Delhi. *H. armigera* larvae were also collected from fields of Central India with special emphasis on the cotton growing districts of Gujarat. Field collected populations (on chickpea and pigeon pea) from Nagpur, Amravati, Yavatmal, Washim, Hingoli, Nanded, Latur, Beed, Aurangabad, Jalna, Buldana, Akola, Dhule, and

Parbhani represented Central Indian *H. armigera* population from Maharashtra while populations from Junagadh, Surat, Vadodara, Anand, Ahmedabad, Bhavnagar, Amreli, Porbander, Rajkot, Surendranagar and Baruch (on chickpea and pigeon pea) represented Gujarat while collections made from Indore represented Madhya Pradesh. Larvae were also collected from cotton, pigeon pea and/or chickpea fields of Dharwad (Karnataka), Mehbubnagar, Warangal, Guntur, Hyderabad, Prakasham, Rajendranagar, Khammam and Nalgonda (Andhra Pradesh), Salem, Coimbatore, Sirivilliputur and Perambalur (Tamil Nadu). Larvae were reared on a chickpea based semi-synthetic diet[15] 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. Two day old, white stage larvae were tested at the rate of one larva per well at a total of twenty-four larvae per concentration on semi-synthetic diet incorporating different concentrations of the toxin. MVP II containing 19.6% Cry1Ac was tested at 6 concentrations in diet-incorporated bioassays. Cry1Ac in MVP II is 99% identical to the active toxin region of Cry1Ac expressed in Bt cotton. 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. A total of 17,330 larvae were subjected to Cry1Ac bioassays. Median Lethal Concentration (LC_{50}) presented in Table 1 was derived from log dose probit calculations[16]. IC_{50} values have been presented in Table 2.

Results and Discussion

The geographical variability in *H. armigera* susceptibility levels to Cry1Ac was slightly different as compared to the variability observed in 1999 before introduction of Bollgard. The LC_{50} values ranged from 0.065 μg Cry1Ac/ml of diet (Sriganganagar) to 0.553 μg Cry1Ac/ml of diet (Mansa) for populations of *H. armigera* from North India. The variability in susceptibility across the strains was 8.5 fold. 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 Cry1Ac was reducing over the years.

The LC_{50} values of populations from Central India (other than Gujarat) ranged from 0.069 μg Cry1Ac/ml of diet with populations from Buldana to 0.659 μg Cry1Ac/ml of diet (Jalna). The variability in susceptibility across populations was 9.5 fold. The LC_{50}s of populations of Gujarat ranged from 0.173 μg Cry1Ac/ml of diet with populations of *H. armigera* from Anand to 1.146 μg Cry1Ac/ml of diet with *H. armigera* populations from Baruch. The variability in susceptibility across populations from across Gujarat was just 6.62- fold.

The highest LC_{50} of South Indian population tested was 0.848 μg Cry1Ac/ml of diet with populations from Guntur and the lowest LC_{50} value was 0.057 μg Cry1Ac/ml of diet with *H. armigera* populations from Salem. The variability of the LC_{50} of *H. armigera* populations from South India was a 14.88 fold.

IC_{50} expressed as µg Cry1Ac/ ml of diet represents the concentration of the toxin that prevents 50% of the treated larvae from reaching the third instar (Kranthi *et. al*., 2005). IC_{50} values ranged from 0.012 (Sriganganagar) to 0.124 µg Cry1 Ac/ ml of diet (Mansa) for North India, from 0.025 (Indore) to 0.151µg Cry 1 Ac/ ml of diet (Akola) for Central India and from 0.009 (Salem) to 0.151 µg Cry 1 Ac/ ml of diet (Guntur) for South India. The IC_{50} values ranged from 0.039 (Anand) to 0.201 (Surendranagar). The variability in IC_{50} values ranged from 10.33 fold, 6.04 fold, and 16.77- fold with *H. armigera* populations of North, Central and South India. The variability in IC_{50} values was 5.15 fold across Gujarat.

The LC_{50} values varied by 20.10 fold and the IC_{50} values varied by 22.33 fold as evidenced by bioassays carried out on *H. armigera* populations collected from 49 districts across the country.

The changes in LC_{50} values in 2007 as compared to 2005-06 and 2006-07, did not indicate signs of resistance development in any of the *H. armigera* populations examined. For example, the LC_{50} values that were between 0.110 and 0.374 µg Cry1Ac/ml during 2005 for the populations made from North India appeared to have changed marginally to a range of 0.094 to 0.416 µg Cry1Ac/ml in field populations collected during 2006 and currently stand at 0.065 ug Cry1Ac/ml to 0.553 ug Cry1Ac/ml of diet. The lowest LC_{50} value recorded in three consecutive years was from Sriganganagar of Rajasthan. The highest LC_{50} value for North India was recorded from Bhantinda in 2005 and in 2006 and Mansa followed by Bhatinda in 2007. Variability in the LC_{50} values was 3.4 fold for North Indian populations of *H. armigera* in the year 2005-06 and 8.59 fold in 2006-07 and remained unchanged at 8.5- fold in 2007.

With respect to Central Indian populations of *H. armigera* the highest LC_{50} value of 0.719 µg of Cry1Ac/ ml of diet were observed with populations from Vadodara in 2005 and 2006, except that populations of Surat also demonstrated a high LC_{50}. In 2007 Baruch recorded the highest LC_{50} followed by Surat with Vadodara occupying the third place. The lowest LC_{50} values in Central India was observed with populations of *H. armigera* collected from Buldana in 2005-06 (0.107 µg Cry1Ac/ml of diet), in 2006-07 (0.092 µg Cry 1Ac/ml) and in 2007-08 (0.065 ug Cry1Ac/ml of diet). The published LC_{50} data of *H. armigera* baseline susceptibility to Cry1Ac in India, prior to the introduction of Bt cotton in the country, were within the range of 0.01 to 0.71 µg Cry1Ac/ ml of diet. However changes in the current data indicates disturbance in the baseline susceptibility of *H. armigera* to Cry 1Ac in populations of Gujarat where the highest LC_{50} value of 1.146 ug Cry1Ac/ml of diet was recorded in the country. Till 2006, none of the sites monitored showed LC_{50} values significantly higher than 0.71 ug of Cry 1Ac/ ml of diet.

Resistance monitoring data showed a progressive decline in the overall variability between *H. armigera* populations with reference to their susceptibility to Cry1Ac over the five-year period after the introduction of Bollgard I. The baseline LC_{50} data obtained in 1999 showed 63-fold variability between *H. armigera* populations that reduced to 27 fold in 2002, 12 fold in 2004, 8.9 fold variability in 2005 and 8.28 fold in 2006 that again increased to 20 fold in 2007. A significant increase in the LC_{50} value of Cry1Ac with *H. armigera* populations of Baruch has also contributed to the increase in variability. Availability of populations of *H. armigera* on cotton whether Bt or non-Bt has significantly declined over the years. Normally, two peak larval populations are observed on rain-fed cotton in central India. During the last two years, populations of *H. armigera* in Central India on cotton have been restricted to a small single peak. Populations of *H. armigera* were tested from about 49 locations this year of which 5 populations (10.20 %) demonstrated LC_{50}s lower than the composite LC_{50} of 0.10 µg Cry1Ac/ml of diet. Two populations of *H. armigera* from North India, 2 populations from South India and one population of Central India demonstrated LC_{50} values less than the composite LC_{50} value of 0.10 µg Cry1Ac/ ml of diet, this year.

The data presented in the report do not indicate the onset of resistance but are significant as they indicate the beginning of a progressive decline in the proportion of *H. armigera* populations susceptible to Cry1Ac. CICR currently maintains the database on the changes in geographic variability in susceptibility of *H. armigera* populations, to Cry1 Ac over seven years subsequent to 1999.

Acknowledgements

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Table 1: Variability in median lethal (LC_{50}) baseline susceptibility of *H. armigera* to Cry1Ac

2007-08	**LC_{50}**	**95%FL**	**LC_{90}**	**95% F.L**	**Slope ± SE**	**Chi-sq**	**heterogeneity**
North India							
Hanumangarh	0.130	0.066-0.258	0.895	0.408-4.128	1.528±0.196	7.293	1.459
Sriganganagar	0.065	0.044-0.096	0.369	0.0224-0.783	1.695±0.223	2.377	0.480
New Delhi	0.078	0.037-0.172	0.574	0.245-3.415	1.491±0.190	8.804	1.761
Fatehabad	0.166	0.111-0.25	1.121	0.656-2.519	1.543±0.200	4.958	0.990
Hisar	0.190	0.105-0.354	1.224	0.594-4.723	1.585±0.207	6.147	1.229
Abohar	0.181	0.081-0.427	1.179	0.483-9.430	1.572±0.206	10.266	2.053
Sirsa	0.244	0.145-0.419	1.342	0.708-4.304	1.732±0.237	5.209	1.041
Bhatinda	0.460	0.253-0.895	2.666	1.263-12.329	1.680±0.236	6.494	1.299
Mansa	0.553	0.305-1.141	3.96	1.72-21.95	1.500+0.210	5.960	1.186
Ludhiana	0.296	0.200-0.445	1.818	1.064-4.190	1.625±0.222	3.903	0.780
Central India							
Dhule	0.212	0.109-0.423	1.196	0.560-5.951	1.705±0.232	7.852	1.570
Hingoli	0.181	0.123-0.268	1.053	0.634-2.266	1.674±0.220	3.632	0.730
Parbhani	0.202	0.139-0.294	1.028	0.636-2.155	1.812±0.249	4.307	0.860
Buldhana	0.069	0.047-0.102	0.411	0.248-0.876	1.654±0.217	4.199	0.840
Beed	0.123	0.068-0.226	0.808	0.397-2.930	1.571±0.202	6.006	1.201
Washim	0.231	0.154-0.352	1.621	0.929-3.780	1.513±0.197	3.004	0.600
Nagpur	0.296	0.193-0.496	2.553	1.369-6.705	1.369±0.181	4.843	0.970
Amaravati	0.397	0.263-0.622	2.931	1.606-7.617	1.477±0.204	3.879	0.780
Nanded	0.318	0.213-0.485	2.157	1.239-5.068	1.542±0.206	1.613	0.320
Aurangabad	0.557	0.311-1.125	4.105	1.80-21.61	1.470+0.210	5.590	1.120
Akola	0.628	0.413-1.015	4.68	2.46-13.42	1.47+0.213	3.690	0.740
Jalna	0.659	0.433-1.07	4.931	2.57-14.38	1.470+0.210	4.400	0.880
Latur	0.136	0.068-0.275	0.905	0.409-4.395	1.554±0.199	7.702	1.540
Wardha	0.163	0.07-0.404	1.141	0.447-10.289	1.516±0.194	10.885	2.177
Indore	0.135	0.074-0.251	0.847	0.413-3.222	1.604±0.206	6.371	1.274
Rajkot	0.328	0.212-0.526	2.97	1.562-8.089	1.339±0.178	3.359	0.670
Ahmedabad	0.256	0.174-0.383	1.554	0.918-3.504	1.637±0.222	3.877	0.780
Amreli	0.597	0.394-0.952	4.288	2.298-11.87	1.496±0.215	3.207	0.640
Porbandar	0.388	0.220-0.725	2.116	1.041-8.78	1.740±0.245	6.153	1.230
Bhavnagar	0.371	0.255-0.552	1.989	1.1188-4.489	1.757±0.247	4.721	0.940
Anand	0.173	0.075-0.422	1.149	0.460-10.237	1.558±0.203	10.834	2.167
Bharuch	1.146	0.718-2.06	10.58	4.85-41.34	1.320+0.210	0.300	0.060
Junagarh	0.729	0.430-1.38	4.15	1.98-18.50	1.690+0.250	5.220	1.044
Baroda	0.758	0.500-1.213	5.29	2.83-14.98	1.519+0.226	1.937	0.390
Surendranagar	0.931	0.589-1.620	8.656	4.11-30.849	1.324+0.200	3.300	0.660
Surat	0.99	0.640-1.560	7.48	3.76-24.32	1.460+0.220	2.090	0.420
South India							
Warangal	0.807	0.516-1.373	7.09	3.47-23.52	1.358+0.200	1.370	0.280
Guntur	0.848	0.541-1.450	7.442	3.61-25.15	1.360+0.200	2.030	0.410
Mahbubnagar	0.479	0.320-0.746	3.216	1.787-8.328	1.549±0.219	3.826	0.770
Hyderabad	0.150	0.077-0.301	0.952	0.438-4.555	1.599±0.209	7.647	1.529
Prakasam	0.302	0.195-0.485	2.863	1.506-7.717	1.313±0.172	3.940	0.790
Rajendranagar	0.343	0.233-0.514	2.021	1.188-4.652	1.660±0.231	2.005	0.400
Khammam	0.270	0.184-0.402	1.576	0.940-3.512	1.673±0.228	2.014	0.400
Nalgonda	0.219	0.148-0.330	1.403	0.823-3.153	1.590±0.208	4.589	0.920
Salem	0.057	0.037-0.088	0.491	0.280-1.146	1.375±0.179	3.501	0.700
Perambalur	0.084	0.057-0.123	0.481	0.293-1.012	1.688±0.220	3.846	0.770
Coimbatore	0.096	0.065-0.143	0.599	0.359-1.291	1.612±0.207	4.705	0.940
Sirivilliputtur	0.116	0.061-0.225	0.799	0.373-3.367	1.529±0.194	6.786	1.357
Dharwad	0.156	0.069-0.371	1.162	0.461-9.258	1.468±0.188	9.926	1.985

Table 2: Variability in Inhibition concentration (IC_{50}) baseline susceptibility of *H. armigera*

2007-08	**IC_{50}**	**95% FL**	**IC_{90}**	**95% FL**	**SLOPE ± SE**	**Chi-sq**	**heterogeneity**
North India							
Hanumangarh	0.028	0.018-0.043	0.212	0.123-0.500	1.463±0.208	2.775	0.55
Ganganagar	0.012	0.007-0.018	0.070	0.043-0.165	1.659±0.283	1.185	0.24
New Delhi	0.014	0.009-0.021	0.083	0.050-0.191	1.649±0.269	0.632	0.06
Fatehabad	0.032	0.022-0.047	0.179	0.109-0.388	1.709±0.241	1.934	0.39
Hisar	0.031	0.021-0.045	0.169	0.103-0.364	1.726±0.245	1.368	0.27
Abohar	0.049	0.033-0.073	0.306	0.183-0.664	1.614±0.215	2.590	0.52
Sirsa	0.056	0.037-0.084	0.364	0.216-0.803	1.575±0.207	4.096	0.82
Bhatinda	0.108	0.06-0.195	0.992	0.473-3.528	1.330±0.168	5.030	1.01
Mansa	0.124	0.062-0.249	1.164	0.503-5.573	1.316±0.166	6.519	1.30
Ludhiana	0.088	0.059-0.131	0.559	0.335-1.205	1.598±*0.206*	3.969	0.79
Central India							
Dhule	0.045	0.030-0.067	0.289	0.172-0.635	1.580±0.212	3.714	0.74
Hingoli	0.046	0.031-0.069	0.313	0.184-0.696	1.544±0.205	3.321	0.66
Parbhani	0.035	0.023-0.052	0.208	0.125-0.457	1.652±0.230	2.690	0.54
Buldhana	0.018	0.012-0.026	0.101	0.062-0.226	1.703±0.263	1.121	0.22
Beed	0.021	0.013-0.032	0.150	0.088-0.354	1.506±0.225	3.022	0.60
Washim	0.065	0.036-0.120	0.413	0.204-1.510	1.602±0.209	6.146	1.229
Nagpur	0.067	0.043-0.102	0.581	0.329-1.361	1.362±0.175	4.008	0.80
Amaravati	0.069	0.045-0.104	0.504	0.293-0.1.134	1.478±0.190	2.642	0.53
Nanded	0.097	0.054-0.175	0.63	0.315-2.174	1.578±0.202	5.814	1.16
Aurangabad	0.106	0.059-0.191	0.687	0.342-2.392	1.577±0.201	5.804	1.16
Akola	0.151	0.075-0.308	0.929	0.423-4.726	1.622±0.212	8.136	1.63
Jalna	0.135	0.070-0.267	0.927	0.426-4.161	1.534±0.197	7.111	1.42
Latur	0.027	0.018-0.041	0.169	0.101-0.379	1.620±0.232	2.671	0.53
Wardha	0.031	0.020-0.047	0.218	0.128-0.501	1.522±0.214	3.372	0.67
Indore	0.025	0.016-0.037	0.181	0.105-0.426	1.478±0.215	2.254	0.45
Rajkot	0.072	0.047-0.108	0.509	0.297-1.132	1.505±0.192	3.822	0.76
Ahmedabad	0.060	0.039-0.091	0.460	0.266-1.046	1.444±0.187	3.384	0.68
Amreli	0.079	0.054-0.116	0.431	0.264-0.899	1.744±0.230	3.648	0.73
Porbandar	0.089	0.058-0.136	0.718	0.412-1.644	1.414±0.179	4.729	0.95
Bhavnagar	0.093	0.059-0.145	0.919	0.504-2.256	1.286±0.162	3.285	0.66
Anand	0.039	0.025-0.058	0.267	0.157-0.598	1.525±0.206	2.270	0.45
Bharuch	0.150	0.098-0.234	1.323	0.733-3.196	1.358±0.171	4.993	1.00
Junagarh	0.176	0.144-0.274	1.574	0.864-3.887	1.346±0.171	2.580	0.52
Baroda	0.152	0.100-0.234	1.232	0.697-2.891	1.410±0.179	2.529	0.51
Surendranagar	0.201	0.131-0.315	1.764	0.966-4.387	1.359±0.174	3.490	0.70
Surat	0.183	0.120-0.283	1.524	0.849-3.685	1.319±0.177	4.280	0.86
South India							
Warangal	0.141	0.054-0.394	1.171	0.414-15.708	1.396±0.177	12.286	2.46
Guntur	0.151	0.068-0.347	1.016	0.421-7.467	1.548±0.201	9.919	1.98
Mahbubnagar	0.077	0.044-0.134	0.525	0.269-1.668	1.534±0.197	5.161	1.03
Hyderabad	0.026	0.017-0.039	0.168	0.100-0.358	1.572±0.227	4.854	0.97
Prakasam	0.081	0.054-0.121	0.519	0.311-1.118	1.588±0.205	3.663	0.73
Rajendranagar	0.058	0.039-0.085	0.353	0.213-0.756	1.626±0.214	4.991	1.00
Khammam	0.092	0.064-0.135	0.482	0.299-0.991	1.788±0.237	2.497	0.50
Nalgonda	0.054	0.036-0.081	0.364	0.216-0.800	1.547±0.202	4.479	0.90
Salem	0.009	0.006-0.018	0.038	0.024-0.088	2.083±0.386	0.796	0.16
Perambalur	0.017	0.010-0.025	0.116	0.069-0.276	1.516±0.238	1.082	0.22
Coimbatore	0.016	0.010-0.024	0.094	0.057-0.214	1.669±0.263	0.526	0.11
Sirivilliputtur	0.024	0.016-0.035	0.148	0.089-0.332	1.618±0.237	1.639	0.33
Dharwad	0.040	0.027-0.059	0.228	0.139-0.493	1.703±0.235	4.262	0.85