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Baseline susceptibility of the American bollworm, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* Berl var. *kurstaki* and its endotoxins in India

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Baseline susceptibility of larvae of the American bollworm, *Helicoverpa armigera* (Hübner) to *Bacillus thuringiensis* Berl var. *kurstaki* was studied by a diet incorporation method. Ninety-six hour median lethal concentrations (LC_{50}) of *Bt* var. *kurstaki* strains and parasporal crystal toxins varied widely for neonate larvae of different populations. Insect populations from nine locations in India showed differences in their susceptibility to *Bt* 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. Insect populations obtained from pigeon pea crops at Navsari from December 2000 to January 2001, and at Delhi from October 1998 to November 2000 showed temporal variation in their susceptibility to *Bt* var. *kurstaki* HD-1 and HD-73. Temporal variation in insect susceptibility was correlated with temperature at these two locations. Insect acclimation to pre-treatment temperature influenced the susceptibility of the F_1 generation to *Bt* var. *kurstaki*. An increase in ambient temperature (about 10°C) increased the susceptibility to *Bt* var. *kurstaki* HD-73 by 7.5-fold. The role of selection pressure, host-plant, xenobiotic and other agroecological conditions on the susceptibility of *H. armigera* is discussed in relation to development of tolerance/resistance and integrated pest management.

BACILLUS thuringiensis (*Bt*) is a spore-forming, Gram-positive bacterium of ubiquitous occurrence, with as many as 50 serotypes or 63 serovars[1]. It produces proteinaceous crystal (Cry) toxins, which are activated by proteases in the alkaline conditions of the midgut. These activated toxins bind with receptors on the brush border membrane vesicles of the midgut epithelium and perforate the cell membrane, which leads to ionic imbalance and eventual insect death[2]. The *Bt* Cry toxins are grouped into 45 classes; many possessing insecticidal-specific insecticidal activity, viz. Cry1, Cry9 (Lepidoptera), Cry2 (Lepidoptera and Diptera), Cry3, Cry7, Cry8, Cry14 (Coleoptera), Cry 4, Cry10, Cry11 (Diptera) and Cry5, Cry6, Cry12-14 (nematodes) (http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/toxins2.html; http://bgsc.org). *Bt* is an effective insecticide, relatively harmless to natural enemies, safe to the higher animals; and environmen-

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tally acceptable[3]. Presently, *Bt* forms about 90% of the world bioinsecticide market.

In India, *Bt* is registered for insect pest management in agriculture and public health. The present usage of about 120 tonnes per annum of *Bt* is likely to increase significantly in view of its recommendation as a component of integrated pest management for agricultural crops[4,5]. Since *Bt* is amenable to genetic engineering, *Bt* transgenic crops like cotton, chickpea, and cole crops are being developed and are in various stages of commercialization in India as elsewhere in the world[6]. Further, new strains of *Bt* with enhanced activity, altered host range and persistence are also being developed to increase its usefulness[7].

Two insect pests targetted by *Bt* as a conventional insecticide as well as through transgenic technology are the diamondback moth, *Plutella xylostella* Linn. and the American bollworm, *Helicoverpa armigera* (Hübner). The diamondback moth is a major pest of cole crops all over the world. It has developed resistance to almost all kinds of insecticides, including *Bt* under field conditions[8,9]. The American bollworm is a polyphagous insect pest of worldwide occurrence. In India, it is reported to cause crop damage worth about US $1 billion per annum. It is a major pest of cotton, pulses and some vegetables. The pest has defied many conventional insecticides by developing resistance[10,11], but there is potential for using *Bt* against it, both as a conventional insecticide and through expression of its toxins in transgenic plants.

It is necessary to study the toxicity of *Bt* and its Cry toxins against *H. armigera* in order to rationalize its use. The toxicity of *Bt* and of *Bt* transgenic cotton against *H. armigera* has been reported earlier[12–21]. In our earlier studies[22], we have extensively reported the toxicity of *Bt* var. *kurstaki* HD-1 and HD-73 to different populations of *H. armigera* collected from various locations in the country by discriminating dose bioassays and found a wide variation in their susceptibility. Since *Bt* usage against *H. armigera* has been low in India, the baseline susceptibility of *H. armigera* should be natural and relatively unaffected by exposure to it. The baseline susceptibility of different populations of *H. armigera* will help in providing a database for developing transgenic crops with the right kind and amount of Cry toxin expression, and would also serve to monitor spatial and temporal development of resistance in target insect species, which is a primary regulatory requirement for transgenic crop technology[23].

The present communication therefore reports on the baseline susceptibility of *H. armigera* to various Cry toxins and comments on the possibility of development of resistance in the test insect.

H. armigera were collected as larvae from agricultural crops in Akola (20 42N, 77 0E), Amravati (20 54N, 77 42E), Bathinda (30 12N, 74 54E), Bharuch (21 42N, 73 0E), Delhi (28 36N, 77 12E; IARI), Guntur (16 18N, 80 24E), Mansa (30 0N, 75 40E), Muktsar (30 30N, 74 50E), and Navsari (20 54N, 72 54E) in India and maintained in the laboratory on a chickpea-based semi-synthetic diet[24] at 27 (± 2)°C and 60–80% RH. The adults emerging from pupae were offered 10% honey solution fortified with multivitamins throughout their egg-laying period. About five pairs of adults were kept in each jar. The eggs were laid on markin cloth moistened with water and were kept in separate jars at 27°C. Neonate larvae were used for bioassays.

Acetone powders of the spore and crystal complex of *Bt* strains HD-1 (4D4) and HD-73 (T03A001), originally received as gift from *Bacillus* Genetic Stock Center, Ohio State University, Columbus, USA and Pasteur Institute, Paris, France respectively, were prepared using the procedure described by Dulmage *et al*[25]. Cells were cultured in nutrient broth at 30°C for 96 h and were harvested by centrifugation at 7000 *g* for 10 min at 4°C. The pellet was washed with 0.5 M sodium chloride and two times with sterile distilled water to remove exoprotease activity. The pellet was re-suspended in 6% lactose solution (at 1/10–1/20 the volume of original broth) and stirred continuously for 30 min. Four volumes of ice-cold acetone were added slowly and stirred for another 10 min. The mixture was then filtered through Whatman No 1 filter paper, dried in partial vacuum and stored at –4°C till further use.

Pure toxins of Cry1Aa, Cry1Ab and Cry1Ac were prepared from recombinant *Escherichia coli* JM 103 strains containing hyper-expressing recombinant plasmid vectors pKK223-3 (BGSC ECE52 *cry1Aa*, ECE53 *cry1Ac* and ECE54 *cry1Ab*) (gifts from *Bacillus* Genetic Stock Center) following the procedure described by Lee *et al*[26]. *E coli* cells were grown in nutrient broth containing 50 µg/ml ampicillin at 37°C for 72 h and were harvested by centrifugation at 7000 *g* at 4°C (3K18, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) for 10 min. Cells were suspended in lysis buffer (50 mM Tris, 50 mM ethylene diamine tetraacetate, 15% sucrose, lysozyme at 2 mg/ml, pH 8.0) and incubated for 4 h. After incubation, lysis buffer was replaced with Crystal Wash-1 (0.5 M sodium chloride and 2% Triton X-100) and sonicated for 3min (Labsonic L, B Braun Biotech International GmbH, Melsungen, Germany) on ice. The pellet was collected by centrifugation at 7000 *g* and washed three times with Crystal Wash-1, three times with Crystal Wash-2 (0.5 M sodium chloride) and three times with sterile distilled water. Finally the pellet was solubilized in buffer (50 mM sodium carbonate, 10 mM dithiothreitol, pH 10.5) at 37°C for 6 h. The supernatant containing the toxin was collected following centrifugation at 7000 *g* at 4°C for 10 min and stored at –20°C till further use.

A known amount of acetone powder of HD-1 and HD-73 was dissolved in the solubilizing buffer, then sonicated for 3 min, incubated at 37°C for 6 h, and the toxin solution stored in small aliquots at –20°C until further use.

The toxin preparations were separated on discontinuous sodium dodecyl sulphate–polyacrylamide gel electrophoresis[27] with an 8% resolving gel. The toxin bands of the samples were identified by comparing with known protein

molecular weight markers. Protein was quantified by eluting the Coomassie Brilliant Blue R-250 visualization dye from the bands, and using bovine serum albumin as a standard as described by Ball[28]. The endotoxin contents of the *Bt* preparations were HD-1 1.243 $\mu g\,mg^{-1}$, HD-73 0.668 $\mu g\,mg^{-1}$, Cry1Aa 1.2 $\mu g\,\mu l^{-1}$, Cry1Ab 1.55 $\mu g\,\mu l^{-1}$ and Cry1Ac 1.25 $\mu g\,\mu l^{-1}$. The viable spore counts per 100 mg of acetone powder of HD-1 and HD-73 were 89.3×10^{10} and 147×10^{10} respectively, by spread plate counting.

Bioassays were carried out by a diet incorporation method as described by Gujar *et al.*[22] using toxin solutions of Cry1Aa, Cry1Ab, and Cry1Ac and acetone powders of the spore-crystal complex of *Bt* strains HD-1 and HD-73. Cry1Aa, Cry1Ab, and Cry1Ac toxin solutions were thoroughly mixed with a known weight of semi-synthetic diet at room temperature. Different concentrations of HD-1 or HD-73 acetone powders in water were added to the diet during cooling (40°C). In both cases the diets were mixed thoroughly and poured into small plastic containers, each container serving as one replication. About six concentrations ranging from 25 to 6000 $\mu g\,l^{-1}$ diet were used for each bioassay with at least five replications per concentration. Ten neonates were released on each container of treated diet. The control consisted of semi-synthetic diet without toxin. A minimum of 350 neonates was used for each bioassay. The mortality was then pooled for each concentration. Concentrations giving a corrected mortality between 20 and 80% at 96 h were mostly used for calculation of median lethal concentrations (LC_{50}). The control mortality ranged from 0 to 15.8% in all bioassays. Experiments with mortality of above 10% in the control were discarded and repeated. All bioassays were carried out at 27°C and 60–80% RH, unless stated otherwise.

Temporal variation of susceptibility of F_1 neonates from the Delhi parental populations was studied by assaying eight populations collected from week 39, 1998 to week 43, 1999 against HD-1 and six populations collected from week 41, 1999 to week 48, 2000 against HD-73. Similar studies were carried out for insect population sampled thrice from pigeon pea fields at Navsari from week 49, 2000 to week 5, 2001. Insect susceptibility in terms of the LC_{50} of HD-73 against neonates using the bioassay method described above was correlated with maximum and minimum temperatures at the location from which the parental population was collected.

The F_1 generation insects from crops of pigeon pea at Akola and from sunflower in Delhi were reared separately on semi-synthetic diet at different temperatures, viz. 35–37°C, 24–27 and 15–20°C. The pupae were then transferred to a rearing room at 27°C and 60–80% RH. The adults were caged separately and allowed to lay eggs on markin cloth. The neonates were bioassayed against the HD-73 preparation as described above. Larval growth and development was recorded by weighing larvae/pupae individually and also recording larval and pupal developmental periods.

Field-collected populations (F_0) were maintained in the laboratory under three different temperature regimes, viz. cold (a range of 16–21°C with a mean of 18.9°C), ambient (a range of 23–25.5°C with a mean of 24.8°C) and hot (a range of 33–35.5°C with a mean of 34.5°C) and the susceptibility of their neonate F_1 larvae to the HD-73 preparation determined as described above.

The mortality data were analysed using a maximum likelihood programme[29], which incorporates correction for control mortality. Resistance ratios were calculated by dividing the LC_{50} of field population by the LC_{50} of the most susceptible field population. Two populations were considered significantly different in their susceptibility if their 95% fiducial limits did not overlap[30].

The populations collected from four different locations in India differed in their susceptibility to Cry1Aa, Cry1Ab, and Cry1Ac (Table 1). Amongst the Cry toxins tested, Cry1Ac was most and Cry1Aa least toxic. The LC_{50} for

Table 1. Toxicity of Cry toxins of *Bt* var *kurstaki* to neonates of *H. armigera*

Population	Date of collection	Host crop	Date of bioassay	LC_{50} ($\mu g\,l^{-1}$)	Fiducial limit (95%)		Slope ± S.E.
					Lower	Upper	
Cry1Aa							
Delhi	12 Oct. 1999	Pigeon pea	15 Oct. 1999	2600	1353	45823	1.3 ± 0.5
Palam	11 Nov. 1999	Cauliflower	10 March 2000	384	233	553	1.7 ± 0.4
Amravati	18 Nov. 2000	Pigeon pea	23 Dec. 2000	4050	2142	42000	1.2 ± 0.4
Akola	25 Nov. 2000	Pigeon pea	20 Dec. 2000	574	447	707	3.1 ± 0.6
Cry1Ab							
Delhi	12 Oct. 1999	Pigeon pea	17 Oct. 1999	691	482	1356	1.6 ± 0.5
Palam	11 Nov. 1999	Cauliflower	28 Feb. 2000	54	38	71	2.4 ± 0.4
Amravati	18 Nov. 2000	Pigeon pea	31 Jan. 2001	291	198	375	2.0 ± 0.4
Akola	25 Nov. 2000	Pigeon pea	20 Dec. 2000	431	242	4106	0.7 ± 0.2
Cry1Ac							
Delhi	12 Oct. 1999	Pigeon pea	15 Oct. 1999	206	51	354	1.1 ± 0.4
Palam	11 Nov. 1999	Cauliflower	10 March 2000	23	3	44	1.6 ± 0.4
Amravati	18 Nov. 2000	Pigeon pea	31 Dec. 2000	263	194	338	2.8 ± 0.5
Akola	25 Nov. 2000	Pigeon pea	25 Dec. 2000	372	233	470	3.0 ± 0.6

Cry1Ac toxin ranged from 22.9 (Palam population) to 372 $\mu g\,l^{-1}$ (Akola population). Cry1Ab showed least toxicity to the Delhi population (LC_{50} 691 $\mu g\,l^{-1}$) and toxicity to the Palam population (LC_{50} 54 $\mu g\,l^{-1}$). The population of *H. armigera* collected from Amravati was the most tolerant to Cry1Aa (LC_{50} 4050 $\mu g\,l^{-1}$), while the Palam population was the most susceptible (LC_{50} 384 $\mu g\,l^{-1}$).

The toxicity of the spore–crystal complex of HD-73 to the different populations ranged from 22 to 123 $\mu g\,l^{-1}$. The Palam population was the most susceptible (LC_{50} 22 $\mu g\,l^{-1}$), whereas the Delhi population (from IARI farm) was the least susceptible (LC_{50} 123 $\mu g\,l^{-1}$). The HD-1 preparation showed about 14.1-fold variation in its toxicity, with LC_{50} varying from 35 (Delhi population) to 494 $\mu g\,l^{-1}$ (Amravati population) (Table 2). The toxicity of the Cry toxins and the spore–crystal complexes of HD-1 and HD-73 ranged widely, viz. Cry1Aa 10.5, Cry1Ab 12.8, Cry1Ac 16.2, HD-1 14.1 and HD-73 5.7-fold.

Temporal variation of susceptibility of F_1 neonates of insects collected from pigeon pea crops at Navsari resulted in a substantial decrease in the toxicity of HD-73 from an LC_{50} of 40 $\mu g\,l^{-1}$ at week 49, 2000 to 271 $\mu g\,l^{-1}$ at week 5, 2001 (Figure 1 *a*). Further, similar temporal variation of susceptibility of F_1 neonates from the Delhi population to *Bt* var. *kurstaki* HD-1 and HD-73 was found to correlate with changes in maximum (not depicted) and minimum temperature from 3 October 1998 to 30 November 2000 (Figure 1 *b*). Insect populations collected in weeks 42 and 43, 1998 showed significantly lower susceptibility compared with that of week 39, but were more susceptible than those collected in week 47 against HD-1. Insect susceptibilities to HD-1 from week 47, 1998 to week 22, 1999 were comparable, although susceptibility was at its lowest in week 13, 1999. The susceptibility decreased till week 43 of 1999 apparently following the decrease in temperature as the winter peaked, and similarly, for increase in insect susceptibility as winter gave way to summer. The insect susceptibility against HD-73 followed a similar trend but not as closely as for HD-1.

Temperature acclimation of insects in the F_0 generation affected the susceptibility of F_1 neonates to *Bt* var. *kurstaki* HD-73. As the temperature regime decreased from 35–37 to 15–20°C for the parental generation, F_1 generation neonates showed a decrease in susceptibility to the *Bt* var. *kurstaki* HD-73 when tested at 27°C (Table 3). This was associated with changes in larval growth and development (Figure 2).

The toxicity of *Bt* var. *kurstaki* HD-73 depended upon the ambient temperature of bioassay (Table 4). There was no significant difference in toxicity of *Bt* var. *kurstaki* HD-73 at cold (16–21°C) and ambient temperature (23–25.5°C), but toxicity was significantly higher under hot conditions (33–35°C).

Perusal of results on toxicity of Cry toxins against neonates of *H. armigera* belonging to different populations showed that Cry1Ac was the most toxic, about 1.7-fold more toxic than Cry1Ab, and 8.8-fold more toxic than Cry1Aa. The higher toxicity of Cry1Ac over other Cry toxins has also been reported in *H. armigera* earlier[16,31,32]. There was a wide variation of about 10.5 to 16.2-fold in the susceptibility of different populations tested for Cry toxins.

Although *Bt* var. *kurstaki* HD-1 based formulations are mostly used for lepidopteran control, HD-73 showed higher

Table 2. Toxicity of *Bt* var *kurstaki* spore–crystal complexes to neonates of *H. armigera*

Population	Date of collection	Host crop	Date of bioassay	LC_{50} ($\mu g\,l^{-1}$)	Fiducial limit (95%)		Slope ± S.E.
					Lower	Upper	
HD-73							
Delhi	12 Oct. 1999	Pigeon pea	18 Oct. 1999	31	5	53	1.3 ± 0.4
Delhi	9 Nov. 1999	Cauliflower	6 Dec. 1999	113	89	140	1.8 ± 0.2
Palam	11 Nov. 1999	Cauliflower	27 Feb. 2000	22	15	27	2.2 ± 0.4
Delhi	3 Nov. 2000	Pigeon pea	14 Nov. 2000	123	90	157	1.6 ± 0.2
Guntur	19 Dec. 1999	Cotton	20 April 2000	72	46	99	1.6 ± 0.3
Bhatinda	23 Oct. 2000	Cauliflower	23 Nov. 2000	43	19	68	1.5 ± 0.3
Akola	25 Nov. 2000	Pigeon pea	19 Dec. 2000	81	62	100	1.7 ± 0.2
Muktsar	7 Nov. 2000	Cotton	29 Dec. 2000	50	39	60	3.3 ± 0.4
Navsari-I	5 Dec. 2000	Pigeon pea	30 Dec. 2000	40	29	49	2.9 ± 0.4
Amravati	25 Nov. 2000	Pigeon pea	1 Feb. 2001	63	53	72	3.5 ± 0.4
Mansa	21 Sep. 2000	Cotton	6 Nov. 2000	69	45	93	1.7 ± 0.3
Bharuch	31 Jan. 2001	Pigeon pea	20 April 2001	91	73	112	2.6 ± 0.4
HD-1							
Delhi	12 Oct. 1999	Pigeon pea	17 Oct. 1999	54	37	74	1.6 ± 0.2
Guntur	19 Dec. 1999	Cotton	2 Jan. 2000	175	115	250	1.5 ± 0.3
Delhi	30 April 2000	Sunflower	14 June 2000	35	22	50	1.5 ± 0.2
Akola	25 Nov. 2000	Pigeon pea	26 Feb. 2001	253	149	574	0.8 ± 0.3
Amravati	18 Nov. 2000	Pigeon pea	3 Feb. 2001	494	304	804	1.2 ± 0.3
Palam	11 Nov. 1999	Cauliflower	8 March 2000	105	75	148	1.1 ± 0.2

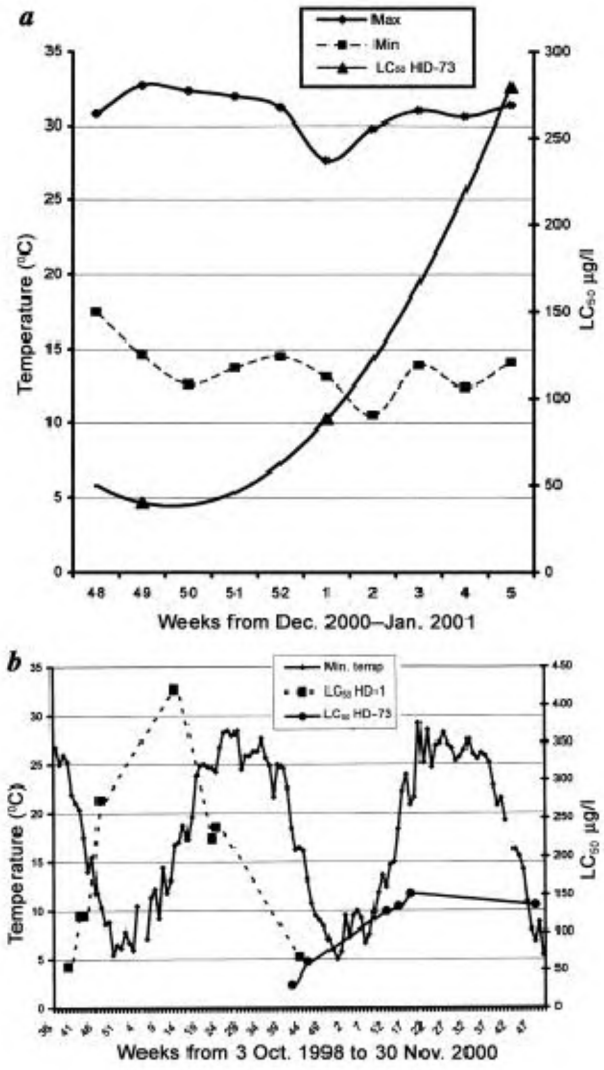

Figure 1. ***a***, Temporal variation in susceptibility of *H. armigera* to *Bt* var *kurstaki* HD-73 at Navsari and ***b***, HD-1 and HD-73 in Delhi (IARI) in relation to temperature (LC_{50} of HD-1 up to 13th week of 1999 are given in Gujar *et al.*[22]).

toxicity than HD-1 by 3.2-fold. There was about 14.1-fold variation in susceptibility of six *H. armigera* populations to *Bt* var. *kurstaki* HD-1. HD-73 showed 5.7-fold variation in toxicity amongst 12 populations studied. The higher variability in toxicity of HD-1 suggests a need for further extensive studies to optimize location specific use of *Bt* since most of the commercial formulations like Biobit®, Dipel®, Biolep®, Halt® are based upon *Bt* var. *kurstaki* HD-1 (a mixture of Cry1Aa (28%), Cry1Ab (53%), Cry1Ac (19%), Cry 2A and Cry2B (<0.1%) developed for use against *H. armigera* and other lepidopteran insects[22,33–35].

HD-73 showed relatively higher toxicity than Cry1Ac to neonates of *H. armigera* despite the fact that the former only contained Cry1Ac. The recombinant *E. coli* expressing Cry1Ac showed wider variability in its toxicity of about 16.2-fold amongst four populations in contrast to 5.7-fold variation in toxicity of HD-73 amongst 14 different populations. The difference in range and intensity of toxicities between *E. coli* Cry1Ac and HD-73 may be attributed to the presence of spores in the latter that might have enhanced its toxicity and lessened the variability. Contribution of spores to the toxicity of *Bt* var. *kurstaki* strains has been reported in *H. armigera*[13], for Cry1Ab and Cry1C in *Ephestia cautella*[36] and Cry1A and Cry1C in *P. xylostella*[37].

The presence of about 16-fold variation in toxicity of Cry1Ac and of similar magnitude for other Cry toxins in the present study compares well with reports elsewhere. Similar ranges of toxicity of Dipel® and purified Cry1Ac protein were reported in 15 geographically diverse populations of *Helicoverpa zea* and *Heliothis virescens* collected from several southern States, Hawaii and the Virgin Islands[38] and in *H. virescens* and *H. zea* in Georgia and South Carolina States[39] to *Bt*. The variability in susceptibility of different populations of *H. zea* (16 to 52-fold) and *H. virescens* (17 to 71-fold) to *Bt* commercial formulations like Javelin WG®, Dipel ES® and Condor OF® was in contrast to that found in respect of Cry1A toxins. The LC_{50} ranges of *Bt* formulations and Cry toxins for field-collected populations were similar to those for laboratory colonies of *H. virescens*, but widely differed for *H. zea*[34]. The high tolerance of *H. armigera* to *Bt* var. *kurstaki* was, however, reported in China, which was attributed to its extensive use and transgenic cotton cultivation[40–43]. In contrast, limited use of *Bt* as a conventional insecticide and an area of about 85,000 ha of *Bt* transgenic cotton out of 9mha in the country do not seem to be enough to act as selection pressure to develop tolerance or resistance in *H. armigera*.

The present study confirms variation in susceptibility of *H. armigera* to HD-1 and HD-73 reported earlier by us[22], and Dhawan and Simwat[44]. The variation in susceptibility to *Bt* var. *kurstaki* toxins may be attributed to the agroecosystem that influences the test insect at physiological level. The significant difference in insect susceptibility for the population collected from the farmer's fields of cauliflower at Palam (a suburb about 12 km from the IARI farm in Delhi) and that on the same crop at the IARI farm shows the importance of history of pest management tactics in influencing *Bt* toxicity. The conventional insecticides used routinely by the farmers seem to enhance insect susceptibility to *Bt*. The resistant/intoxicated insects tend to divert their physiological resources towards meeting fitness costs imposed upon by the selection pressure of conventional neurotoxic insecticides. This makes them more vulnerable to other control agents like *Bt* acting on site other than those belonging to conventional neurotoxicants[45]. Hence, *Bt* was found even more effective against insecticide-resistant *H. armigera*[18,46]. It is therefore essential to know the treatment history on the crop for developing baseline susceptibility studies and monitoring for resistance development[10].

Table 3. Toxicity of *Bt* var *kurstaki* HD-73 spore–crystal complex to F_1 neonates of *H. armigera* reared for a generation at different pre-treatment temperatures

Temperature (°C)	Date of collection	Date of bioassay	LC_{50} (µg l^{-1})	Fiducial limit (95%) Lower	Fiducial limit (95%) Upper	Slope ± S.E.
Insects collected from pigeon pea at Akola						
35–37	25 Nov. 2000	19 Dec. 2000	21	6.0	33	2.7 ± 0.8
24–27	25 Nov. 2000	19 Dec. 2000	81	62	100	1.7 ± 0.2
15–20	25 Nov. 2000	26 Feb. 2001	308	224	565	1.6 ± 0.3
Insects collected from sunflower at Delhi						
35–37	30 April 2000	Aug 21, 2000	18	0.10	36	1.5 ± 0.6
24–27	30 April 2000	June 14, 2000	34	21	45	1.7 ± 0.3

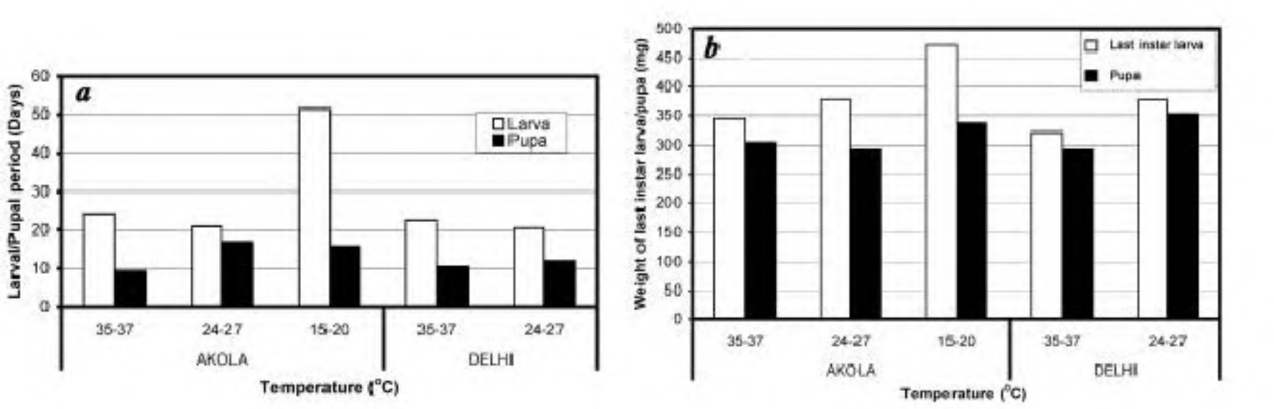

Figure 2. Growth and development of *H. armigera* from two locations, viz. Akola and Delhi at different temperatures. ***a***, Larval and pupal period; ***b***, Larval and pupal weights.

Table 4. Toxicity of *Bt* var *kurstaki* HD-73 spore–crystal complex to neonates of *H. armigera* at different temperatures

Temperature (°C)	LC_{50} (µg l^{-1})	Fiducial limit (95%) Lower	Fiducial limit (95%) Upper	Slope ± S.E.
16–21	96	65	149	1.0 ± 0.2
23–25.5	128	94	192	1.2 ± 0.2
33–35.5	17	8	26	1.9 ± 0.3

The variation in insect susceptibility to xenobiotics depends upon the test insect, the selection regime and the environment with respect to time. The American bollworm, being a highly mobile and polyphagous pest, remains a challenge for interpreting estimates of inter-population variability in *Bt* susceptibility[47]. Among the factors involved, the abiotic factor like temperature and biotic factor like host plant seemed to influence susceptibility of insects to *Bt* var. *kurstaki* significantly. As the crop matured and ambient temperature decreased, the susceptibility of the larvae also decreased. The winter months of December and January seemed to slow the larval growth depending upon temperature. The larvae grew healthier as larval period increased with decrease in temperature, and hence their progeny seemed to develop a good deal of tolerance or resistance to *Bt* var. *kurstaki* HD-73. The role of body weight and size in susceptibility to *Bt* is discussed in the diamondback moth[48]. The susceptibility of *H. armigera* appeared to follow a cyclic pattern, initial decrease as winter progressed, followed by increase in susceptibility in summer, more clearly for HD-1 than HD-73. The pre-treatment temperature acclimation of insect in F_0 generation and the susceptibility of their F_1 progeny investigated under laboratory conditions confirmed the role of pre-treatment temperature acclimation on the susceptibility of the progeny. Besides, the ambient temperature also influenced the efficacy of *Bt*, as observed in the present study. Similar positive correlation of temperature with toxicity of *Bt* has been reported in three species of the apple leafroller[49] and the oblique-banded leafroller[50]; which suggests the importance of seasonal influence under the field conditions. The host crops, as they mature, develop defensive mechanisms in relation to developmental controls[51,52] as well as in response to insect and pathogen attack[53,54]. The induction of protease inhibitor genes in crop plants may lead to intake and accu-

mulation of protease inhibitor by target insect, which might in turn influence insect susceptibility to *Bt*. Besides, protease inhibitors may inhibit insect growth and development by inhibiting midgut proteases involved in digestion. The role of plant phenolics[55], chlorogenic acid and polyphenol oxidase[56], some furanocoumarins in celeriac (*Apium graveolens*)[57] and of secondary plant metabolites[58] in influencing toxicity of *Bt* to insects shows the importance of plant–*Bt* interaction in insect susceptibility. The susceptibilities of *H. virescens* to *Bt* var. *kurstaki* were influenced by host crops[59]. Meade and Hare[60] examined the role of host plant cultivars of celeriac (*A. graveolens* var. *rapaceum*) and environment on the susceptibility of two noctuids, *Spodoptera exigua* and *Trichoplusia ni* to *Bt* var. *kurstaki* NRD-12 spore–crystal complex and its commercial formulation, Javelin®. The efficacy of *Bt* var. *kurstaki* was highest on the resistant plant cultivar compared to the susceptible one. This is due to the general stress that the insect undergoes due to inadequate and/or suboptimal diet. The host plant suitability for both insects decreased with increasing plant age, which affected toxicity of *Bt*. The environmental influences also determined host plant suitability for the two noctuids, which affected toxicity of *Bt*. The LC_{50s} of *Bt* formulations was higher on cotton than on soybean for *H. virescens*[33].

Although the present study showed wide variation in susceptibility to *Bt*, the development of resistance leading to control failures under field conditions will, however, depend up on the presence of initial frequency of the alleles, inheritance of resistance, selection pressure and insect behaviour over a period of time. Considering the moderate level of expression of Cry1Ac in Australian transgenic cotton[61] (of about 0.5–2.9 ppm in terminal leaves) *vis-à-vis H. armigera*, ability of the bollworm to develop resistance under selection pressure[62], and possibility of presence of high level of resistance genes in the Australian population[63]; the resistance problem needs to be addressed discretely in a given ecosystem. It is essential that the baseline monitoring of insect susceptibility should be considered an absolute necessity for resistance management in the country.

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A new species of frog (Ranidae, Rhacophorinae, *Philautus*) from the rainforest canopy in the Western Ghats, India

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A new frog of the genus *Philautus* is described from Wayanad district in the Western Ghats of India. It differs from all its congeners by the combination of webbed fingers, nearly fully webbed toes and a distinct dermal fringe along the outer margin of the fore- and hind limbs. *Philautus nerostagona* sp. nov. exhibits several characteristics suggesting that it is strongly adapted to life in the upper layers of the rainforest.

THE discovery of quite a number of undescribed frog and caecilian species in India[1–4] during the past decade illustrates that our knowledge on the amphibian diversity of this region is still far from complete. While the ground- and shrub level of rainforests in the subcontinent is now being explored intensively, it remains difficult to investigate the lowest (subterranean) and highest layers (canopy) of these habitats. During our exploration of the anurans in the Western Ghats of India, we located an undescribed frog inhabiting the canopy layer (between ca. 10–20 m) of the forests in Kalpatta, Wayanad district. The glandular belly, the large unpigmented eggs and the fully endotrophic development identify this taxon as a member of the genus *Philautus*.

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Philautus nerostagona sp. nov. (The species epithet is the combination of two Greek words – *nero*, water and *stagona*, drop – and refers to the call resembling drops falling down in water.) Holotype: Bombay Natural History Society (BNHS), Mumbai, Maharashtra, India, BNHS 4244, an adult male collected by S.D.B. on 20 July 2000 at an altitude of 1000 m asl, from Kalpatta, 11°38′N, 76°08′E, North of the Palghat Gap, Wayanad district, Kerala, India; Paratypes: BNHS 4245 (adult male), collected by Anil Zachariah on 4 June 1999, and BNHS 4246 (adult male), collected by S.D.B. on 1 August 2000 from the same locality as the holotype.

Diagnosis: *Philautus nerostagona* is easily distinguished from all species in the genus by a combination of the presence of webbing between the fingers, nearly fully webbed toes, a distinct dermal fringe along the outside of the fore- and hind limbs, and a tongue with a pointed papilla.

The description (all measurements in mm) of the holotype (Figures 1 *a* and 2) follows terminology used elsewhere[5]: Small size (SVL 34.0); head (Figure 2 *b*) broader than long (HW 13.7; HL 12.6; MN 10.6; MFE 9.1; MBE 4.8); outline of snout in dorsal view rounded, in profile rounded, its length (SL 5.3) longer than the horizontal diameter of the eye (EL 4.4); canthus rostralis sharp, loreal region obtusely concave; interorbital area slightly concave, equal (IUE 3.2) to upper eyelid (UEW 3.2) and internasal distance (IN 3.2); distance between anterior margins of eyes (IFE 6.8) 1.7 times in distance between posterior margins of eye (IBE 11.7); nostrils oval, closer to tip of snout (NS 1.6) than to front of eyes (EN 3.5); pupils oval, horizontal; tympanum distinct, rounded, its diameter (TYD 2.0) less than half the diameter of the eye, larger than distance from tympanum to eye (TYE 1.0); vomerine teeth absent; tongue large (9.8 × 5.7), emarginate, with a pointed papilla; supratympanic fold distinct, from posterior corner of upper eyelid to base of forelimb; no co-ossified skin on skull.

Forelimbs (FLL 7.4) shorter than hand (HAL 10.6; TFL 6.3; Figure 3 *a*); dermal fringe along the outside of the fore limbs; relative length of fingers: I < II < IV < III; tips of fingers with disks, oval, with distinct circummarginal grooves; fingers, with lateral dermal fringe moderately webbed; subarticular tubercles prominent, rounded, single, III2 and IV2 absent; prepollex rather distinct and oval; supernumerary tubercles distinct, prominent on palm and second and third fingers.

Hind limbs moderately long, heels touch with limbs folded at right angles to the body; shank nearly five times longer (TL 17.1) than wide (TW 3.6), as long as the thigh (FL 17.1), and longer than distance from base of internal metatarsal tubercle to tip of toe IV (FOL 14.3); length of toe IV (FTL 8.8) 2.6 times in distance from heel to tip of toe IV (TFOL 23.1); relative length of toes: I < II < III < V < IV; tips of toes with discs, rather wide compared to the toe width, with a distinct circummarginal groove; toes nearly fully webbed (Figure 3 *b*); a distinct dermal fringe along the outside of the hind limbs, ending with a well-developed spinular projection on the heel; subarticular tubercles distinct,