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3. *Effect of Bt on Non-Target Organisms*

In view of its mode of action, highly specific to lepidopteran insect pests, one of the major advantages of Bt technology is the safety to non-target insects, as well as birds fish, mammals etc. This has been amply demonstrated during the last 10 years of Bt-cotton and Bt-corn cultivation globally.

Advantages to insect pest management, soil health and to human health in particular, due to the introduction of Bt-cotton in China and, thereby, substantial reduction of the use of broad-spectrum chemical insecticides, have been enumerated in some detail by Huang et al. (2002).

We have carried out all the mandatory studies in respect of the effect of Bt cotton on soil microflora, including an investigation pertaining to the presence of Bt gene and protein in cotton soil rhizosphere:

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Effect on Earthworms

Two types of studies were undertaken for earthworms i) Comparison of earthworm population, in soils under Bt-cotton and non-Bt cotton. Such studies were initially carried out during the Multi-Location Trials (2004) at the Isarwadi farm. Between two rows of Bt-cotton plants, and similarly between two rows of non-Bt plants, holes were dug out and the soil was examined for earthworms and other insects. Each hole measured about a meter in depth and 30-35 cm in dia. Three such holes were dug out for each of the test Bt-cotton hybrids and counter-part non-Bt hybrid. This exercise was carried out at the beginning (just before planting the crop) and repeated at 60, 120, 150 and at 225 days after sowing. However, earthworms were almost non-existent at the Isarwadi trial. Therefore, similar study was taken up at our Research Farm at Aurangabad. The soil at this farm is heavier, comparatively richer in organic content and, therefore, expected to be richer in soil biota. However, even here the numbers were very low (please see Table 1).

Table 1. Population of Earthworms in Bt-Cotton Trial Plots at Nath Seeds' Research Farm at Aurangabad

Test Hybrids		Number of Earthworms (±SE) at Days after sowing				
		0	60	120	150	225
NCEH-6	Bt	0.0± 0.6	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.0
NCEH-6	NBt	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NCEH-2R	Bt	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NCEH-2R	NBt	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
NCEH-3R	Bt	0.0 + 0.0	0.5 + 0.5	0.0 + 0.0	2.0 + 1.0	0.0 + 0.0
NCEH-3R	NBt	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 6.0

ii) Since the number of earthworms was small even here, another experiment was initiated. Nucleus culture of earthworms, *Perionyx excavatus*, was obtained from Coimbatore (Tamilnadu). This is a voracious surface feeding prolific species and commonly found in south India. Earthworms were maintained in a dark, cool, moist place and food was supplemented with farm manure and plant biomass. For conducting bioassay, medium sized earthworms with well developed clitellum were utilized.

Soil from the root zone of Bt cotton plants (NCEH-6 and NCEH-2R), as well as from the counter-part non-Bt plants, was collected from the field trial plots of Nath Seeds's Aurangabad Research Farm. Using these soil samples, potting mixtures were prepared using 1 : 1 and 1 : 3 cotton root zone soil (1 part) and normal potting mix (1 or 3 parts), such that two kinds of soil mixtures were prepared; one having 50% and the other having 25% cotton root zone soil. The normal potting mix contains about one-fourth content of farm yard manure.

Large-size (12 inch dia.) pots were filled with the two potting mixtures (~12 kg soil). In each of these pots, six earthworms were released. Before doing so, water was sprinkled on the pot mix so as to moisten the soil. Five pots were used for each replication of a treatment. The average body weight of the earth worms was determined before release and at the end of 30 days period (Table 2).

Table 2: Effect of Bt cotton Root-Zone Soil on Growth of *P. excavatus*.

Treatment	Initial weight (g)	Weight after 30 days
1. NCEH-2R Bt (100%)	10.10 ±0.67	10.20 ±0.55
2. NCEH-2R Bt (20%)	10.00 ±0.71	10.45 ±0.64
3. NCEH-2R NBt (100%)	8.50 ± 0.54	8.80 ± 0.38
4. NCEH-2R NBt (20 %)	8.30 ± 0.32	8.80 ± 0.26
5. NCEH-3R Bt (100%)	11.05 ±0.86	11.30 ±0.75
6. NCEH-3R Bt (20%)	10.30 ±0.92	9.20 ± 0.98
7. NCEH-3R NBt (100%)	9.40 ± 0.74	9.40 ± 0.80
8. NCEH-3R NBt (20%)	10.40 ±0.68	10.40 ±1.03
9. NCEH-6 Bt (100%)	11.00 ±0.93	11.75 ±0.68
10. NCEH-6 Bt (20 %)	10.25 ±0.86	10.70 ±0.55
11. NCEH-6 NBt (100%)	10.70 ±0.91	11.35 ±0.98
12. NCEH-6 NBt (20%)	12.80 ±0.95	13.10 ±1.01
13. Control (normal potting mixture, unammended with cotton soil)	11.9 ± 0.84	12.2 ± 0.88

The results have shown that there was no difference between soil used from Bt cotton rhizosphere to that used from non-Bt plants.

To date, two studies (Ahl Goy et al. 1995; Saxena & Stotzky, 2001a) considered the effect of transgenic Bt corn on earthworms. In a 14-day toxicity study, Ahl Goy et al. (1995) did not find any significant effects of transgenic Bt corn expressing the CryIAb toxin on mortality or weight gain of the epigeic species Eiseria foetida (Savigny). Similarly, in a laboratory experiment, Saxena & Stotzky (2001a) did not find any significant difference in percentage mortality and weight of earthworms after 40 days in soil planted or not planted either with Bt or non-Bt corn or after 45 days in soil amended or unamended with biomass of Bt or non-Bt corn

iii) Using pitfall traps for assessment of Soil Insects

Pitfall trapping has been extensively used as a reliable method of sampling insect populations because of its simplicity and ease of operation. It is an effective and cheap way of qualitatively surveying the grounds and surface active arthropods in different habitats.

In our studies conducted at the Isawadi farm, pitfall traps were made of plastic jars of about 40 cms, sunk deep near the root zone of Bt-cotton test hybrids as well as the counterpart non-Bt hybrids. Opaque plastic covers, raised about an inch off the ground over the trap, positioned appropriately such that trap works as an effective capture of insects, whereas, the cover keep out debris and such other undesirable entry of animals etc. A solution of 10% formalin was placed in the traps. Three such pitfall traps were placed in between two rows of Bt-cotton test hybrids, as well as counter-part non-Bt hybrids, and the tests were conducted at 60, 100, 150 and 225 DAS.

These traps were examined 4 days after installation and the contents examined carefully for the numbers and kinds of trapped insects. Beetles, bugs, thrips, aphids, spiders and crickets were the predominant species, whereas, Collembola were very few in numbers in all the traps, irrespective of the stage of crop growth or the test hybrid, Bt or non-Bt. The relative abundance of these trapped insects did not not show any differences between Bt or non-Bt hybrids (Table 3).

A particular survey was done at the Aurangabad Reseach farm for comparative evaluation of the relative abundance of Collembola, given its sensitivity to pesticidal residues in soil. However, even here, the number of Collembola collected in the traps was very few and no differences were seen between Bt and non-Bt hybrids.

Table 3: Comparative assessment of the relative abundance of soil insects trapped at Aurangabad and Isarwadi Bt-cotton field.

Insect Sampling (Days after Sowing)	Test Hybrid	Soil Insect Population (Mean no. of insects/ treatment)	
		Aurangabad	Isarwadi
60 DAS	NCEH-2R Bt	1.50	0.00
	NCEH-3R Bt	0.00	0.50
	NCEH-6 Bt	1.00	2.00
	NCEH-2R NBt	0.50	0.50
	NCEH-3R NBt	0.00	2.00
	NHH-44 NBt	1.00	1.00
	±SE	0.55	0.76
100 DAS	NCEH-2R Bt	0.00	0.00
	NCEH-3R Bt	0.00	0.00
	NCEH-6 Bt	0.50	0.00
	NCEH-2R NBt	0.50	0.50
	NCEH-3R NBt	0.00	0.00
	NHH-44 NBt	0.00	0.00
	±SE	0.23	0.18
150 DAS	NCEH-2R Bt	0.00	0.50
	NCEH-3R Bt	1.00	0.00
	NCEH-6 Bt	0.50	1.00
	NCEH-2R NBt	0.00	0.50
	NCEH-3R NBt	0.50	0.50
	NHH-44 NBt	1.00	0.00
	±SE	0.40	0.34
225 DAS (Post harvest)	NCEH-2R Bt	0.50	0.00
	NCEH-3R Bt	0.50	0.50
	NCEH-6 Bt	2.00	1.00
	NCEH-2R NBt	1.00	0.00
	NCEH-3R NBt	0.00	0.50
	NHH-44 NBt	2.00	0.50
	±SE	0.76	0.34

iv) Effect of Bt cotton on Nematodes

Post-harvest, at 225 days after sowing of the Bt cotton trial plot, soil samples collected from in between rows of Bt cotton and non-Bt cotton test plots, were assayed for nematode communities. No differences were observed in the total nematode populations.

Table 4: Post Harvest Nematode Populations at Aurangabad and Isarwadi in Bt Cotton Fields.

Treatment	Nematode Population / 100 g Soil.	
	Aurangabad	Isarwadi
NCEH-2R Bt	361	216
NCEH-3R Bt	336	235
NCEH-6 Bt	323	198
NCEH- 2R NBt	311	224
NCEH-3R NBt	352	206
NHH-44 NBt	328	244

Sucking Pests

Till 30 days after sowing (DAS), no significant presence of sucking pests was noticed in any of the materials tested, Bt or non-Bt. Beyond that, the incidence of such insects was found to increase rapidly. At about 45 DAS, the sucking pest pressure developed to a stage of crossing the economic threshold level. In particular, the damage inflicted by serpentine leaf miner was the most severe. At this point, it was considered appropriate to apply Confidor @ 0.5 ml per liter. This single sucking pest-specific application effectively checked any further damage and this was the only pesticidal spray used. Table 3 presents the relative population pressure of different sucking pests at 30, 45 and 60 days of crop growth. Though in most cases, the incidence of sucking pests had reduced significantly by 90 DAS, however, in the case of MAHYCO's MECH-12, the sucking pest pressure, especially that of aphids and jassids,

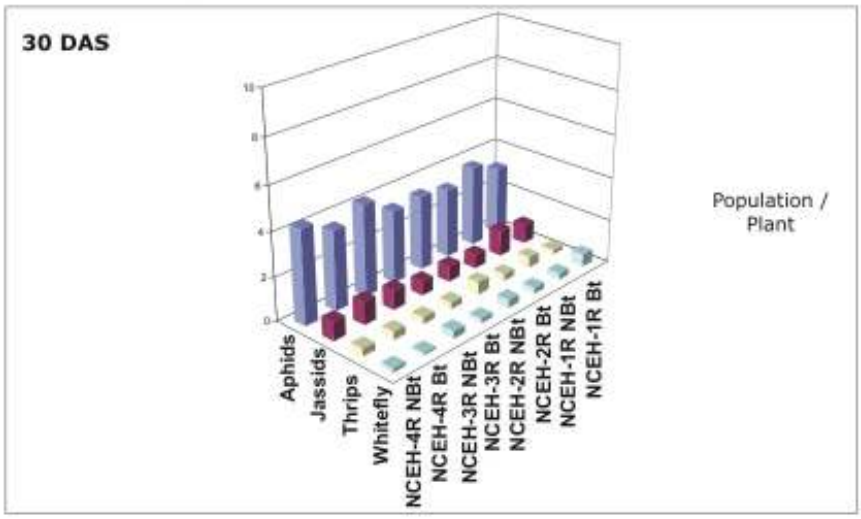
continued and was recorded highest at 60 DAS. However, this may be due to the genotypic susceptibility of this hybrid rather than due to the presence of Bt transgene. In the two other MAHYCO Bt hybrids, no such difference in susceptibility between Bt hybrids and non-Bt counterparts was observed Over all, no significant differences were observed among different test materials, whether Bt or non-Bt.

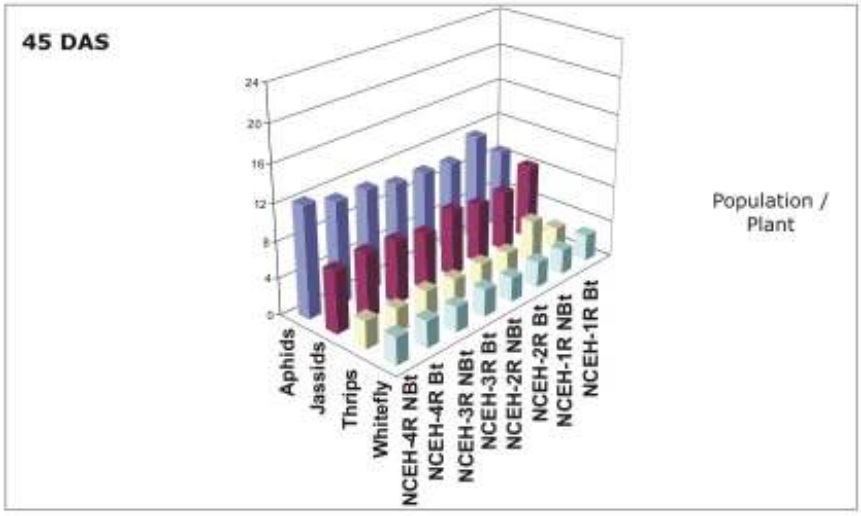
White fly are found mostly on the ventral leaf surface. Observations recorded include counting all the nymphs and adults per randomly selected leaf; one from the upper region of the plant canopy, the other from the middle region and the third from the lower area.

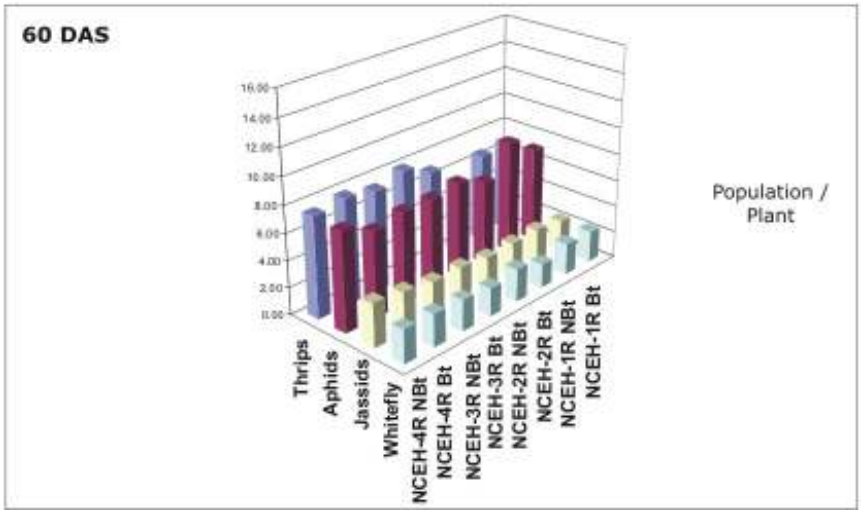
Adults and nymphs of Aphids, as well as Jassids and Thrips are found mostly on the ventral leaf surface. Observations included counting all the nymphs and adults per randomly selected leaf; one from the upper plant area, the other from the middle region and the third from the lower area.

Multiple-Location Field Trial (2004) Central Zone

Population of Sucking Pests on Bt, Non-Bt and Check Hybrids

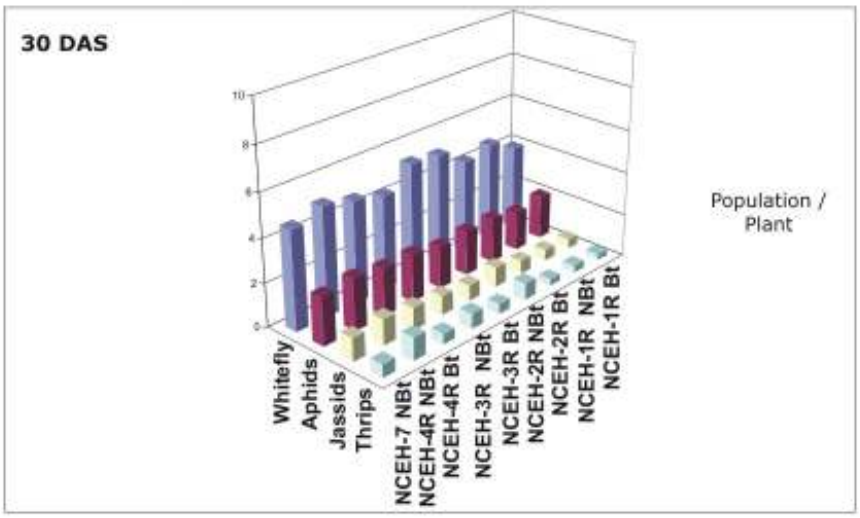


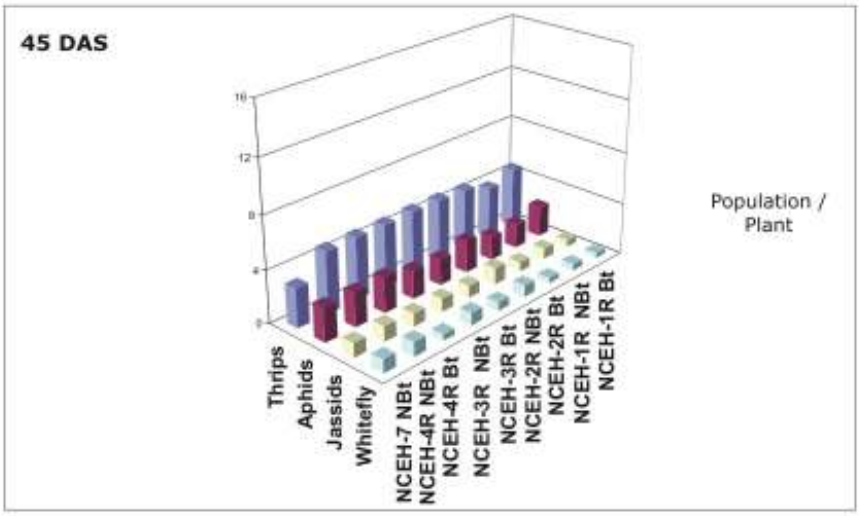


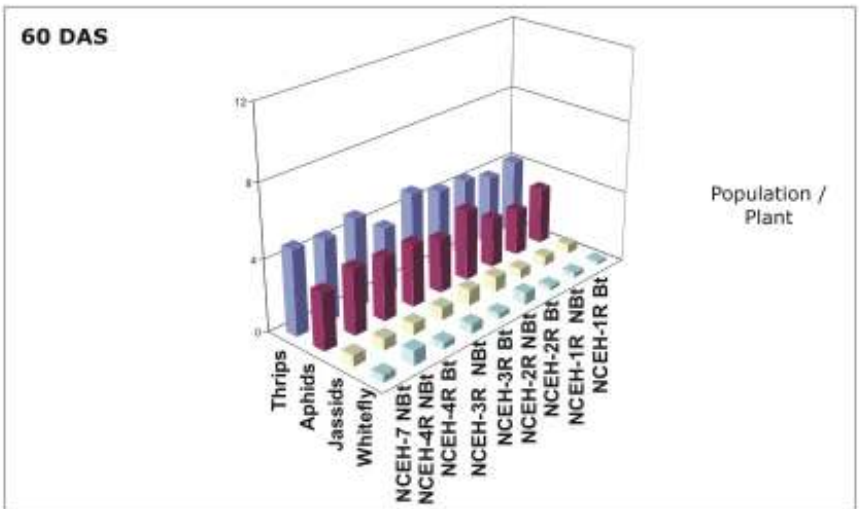


Multiple-Location Field Trial (2004) South Zone

Population of Sucking Pests on Bt, Non-Bt and Check Hybrids

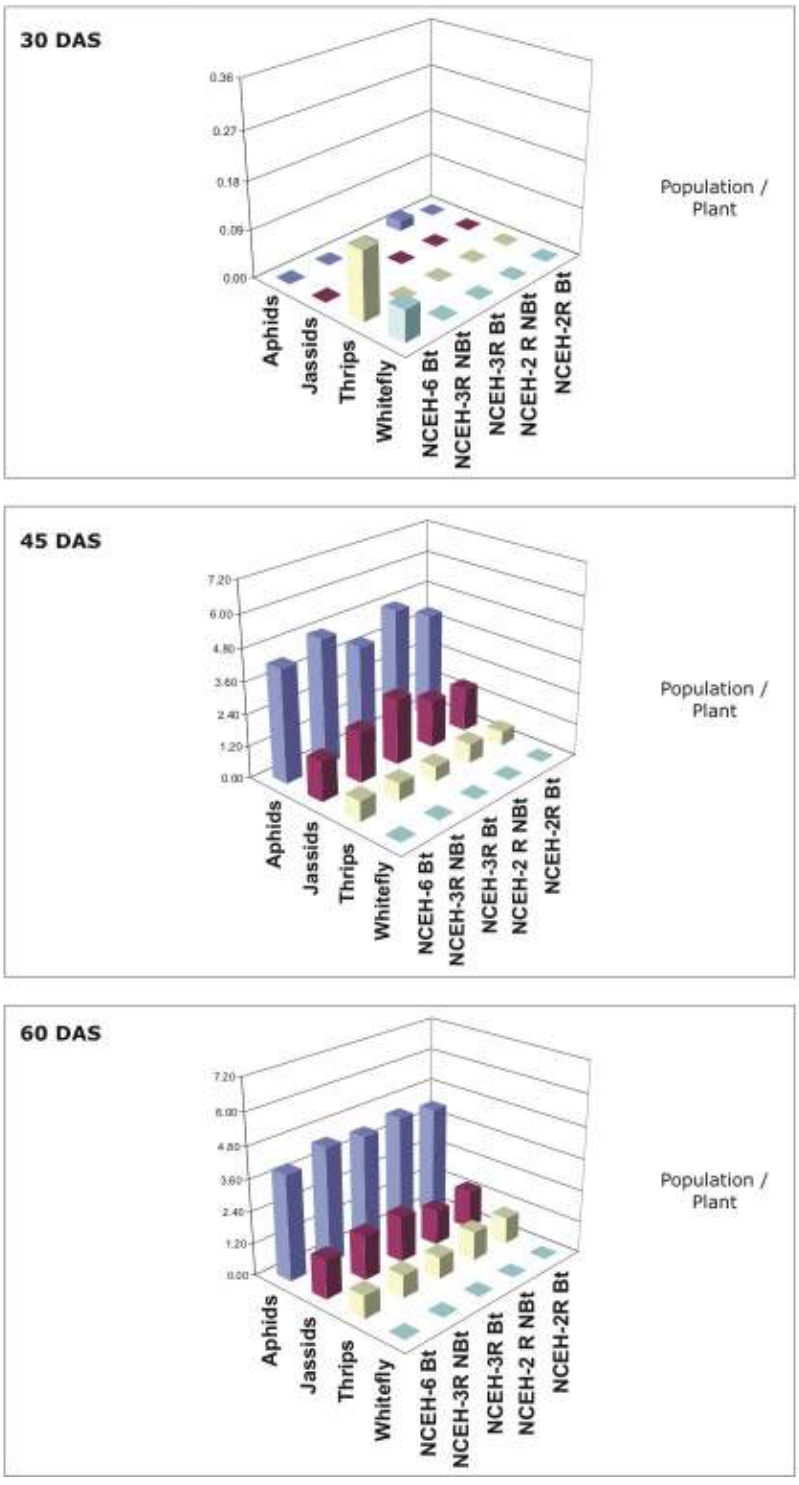






Multiple-Location Field Trial (2004) North Zone

Population of Sucking Pests on Bt and Non-Bt Hybrids

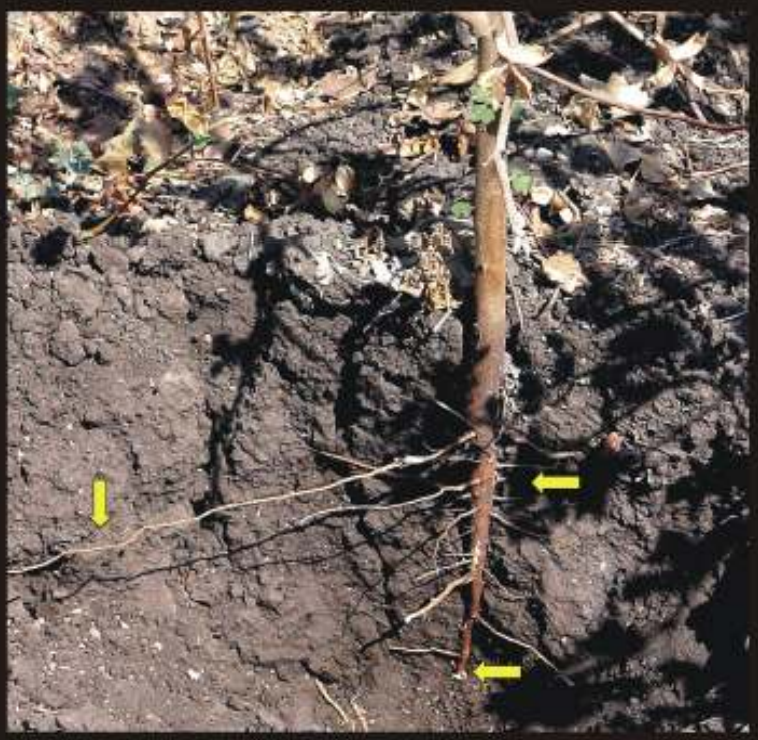


i) Presence of Bt gene and Bt protein in soil and roots:

Using forward and reverse primers specific to the structural Bt-gene sequences, PCR techniques were employed to detect any evidence of Bt gene in the soil.

Soil samples (~100 g) were collected from different depths (20-45 cm) of Bt-cotton (test hybrids) root zone (see photo), at different stages of growth (60, 120, 150 and 225 days after seed sowing). Such soil samples were collected from Bt-cotton hybrids trial plots at Isarwadi (~35kms from Aurangabad and also from Aurangabad Research Farm. Soil samples were also collected from Green House raised plants (Bt protein expression is relatively higher in such plants).

Arrows Indicate Specific Locations from where Soil Samples were Collected



Soil Samples (at arrows) and Terminal Leaf Samples were drawn from Bt Cotton Plants (60 day-old)

Soil samples were sieved and then 10 g representative sample, from each level of root-zone collection, thoroughly mixed with 90 ml distilled water. Supernatants were used for PCR based Bt-gene detection and also analysed immunologically for presence of Cry1Ac/Ab Bt protein using DesiGen (Jalna, Maharashtra) 96-well Quant-T ELISA kits for detection as well as quantitative estimation of the Bt protein. No larvicidal studies were undertaken with any of the soil samples since all soil samples tested negative for Bt protein.

No evidence of Bt gene presence or any detectable levels of Bt protein was observed in any of the large number of soil samples tested. However, some isolated cases of very low level Bt protein was detected in soil samples collected from very close to the main roots of Bt cotton plants at 90-120 days of age. However, it would be difficult to conclude whether this was due to normal root exudation or possibly some exudation due to injury or due to inclusion of some dead root surface cells.

On the other hand, Bt protein was detected in all the samples of roots tested, whether from Bt cotton of 60 DAS or as old as 225 days. In the latter case, whereas the plants had all dried except for some tiny green leaves here or there, the roots tested positive but the tiny leaves did not show any detectable presence of Bt protein.

In respect of the lack of detectable levels of Bt protein in Bt-cotton soil samples, our results are in conformity to those of Saxena et al (2004). In their study, soil and hydroponic solution in which Bt corn, rice, or potato had been grown were both immunologically positive for the presence of the Cry proteins and toxic to the larva of *M. sexta* (corn and rice) and *L. decemlineata* (potato). No Cry proteins were detected immunologically or by larvicidal assay in any soil or hydroponic solution in which Bt canola, cotton, or tobacco, as well as all near-isogenic non-Bt plant counterparts or no plants, had been grown. However, the Cry proteins were detected in the tissues of all Bt plants. There were apparent differences in exudation of the proteins (as evaluated immunologically and by mortality and weight of surviving larvae) between plant species.

In the case of Bt-corn where root exudates of Bt protein are known to occur all through the duration of crop, a very extensive and intensive study was conducted recently by the European Union (Griffiths et al. 2005). Field trials were established and monitored at three European sites (Denmark, Eastern France and South-West France). The concluding statement of this French-British-Danmish multi-Institutional project was: "The effect of the Bt maize was small and within the normal variation expected in these agricultural systems".

5. *Base Line Susceptibility of Helicoverpa armigera*

Procedure:

Laboratory cultures were established by collecting about 300 late instar larvae of *Helicoverpa armigera* from the cotton field in the following locations in the cotton belt of North, South and Central India. Abohar (Punhjab), Guntur (A.P), Rajkot and Vadodara (Gujarat), Isarwadi, Jalgaon and Yeotmal (M.S.).

The larvae were reared on a semi synthetic diet, which contains chickpea as its main component. Larvae were inspected regularly to ensure that they remained pathogen-free. The colony was maintained in a culture room with a mean temperature of 28ºC, 60% RH and with a photoperiod of 14:10 (L:D). About 200 moth representing each location were obtained. Each mating cage contained about 50 moths and eggs were collected daily. These eggs were surface sterilized in 0.05% sodium hypochlorite solution and incubated for hatching. The F1 generation neonate larvae were used for bio-assays. The Bt protein was assayed by diet-incorporation method. Seven different concentrations ranging from 0.02 to 8 µg/ml of diet were used. Newly hatched, active larvae were transferred onto the solidified diet in the 24-cell insect-rearing tray with a fine hair brush (1 larvae / well). After larval transfer, insect-rearing trays were covered with semi-permeable wrap and lead was closed. Each treatment was replicated three time and at least 20 larvae formed one experimental unit. Mortality of larvae was scored every 24 hrs. for seven days. The larvae were marked dead when they did not move when prodded. The surviving larvae were severely inhibited and were weighted on the final day of experiment. Each bio-assay was repeated two times. In each experiment,