Transgenic cotton expressing the Bacillus thuringiensis endotoxin : effects on soil microflora & non-target soil organisms with special reference to earthworms

Chapter V

Annexure 2

Effect of Bt Cotton on Soil Microflora and Beneficial and Non-Target Soil Organisms

Study on the safety of Bt protein produced in field grown transgenic cotton on naturally occurring soil organisms in five locations

Protocol – 4 Report

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Study title

Transgenic cotton expressing the *Bacillus thuringiensis* endotoxin: Effects on soil microflora and non-target soil organisms with special reference to earthworms

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Abstract. An important aspect of the biosafety assessment of genetically engineered (transgenic) plants is to study their impact on soil ecosystem including changes in the plant-associated microflora. In the described research we have evaluated the impact of the *Bacillus thuringiensis* endotoxin on soil microflora and also on earthworms. Soil samples were collected periodically from *Bt* and non *Bt* plots of five different locations (Yeotmal in Maharashtra, Barwah in Madhya Pradesh, Kallakal in Andhra Pradesh, Kovilpatti in TamilNadu and Harpanhalli in Karnataka) where transgenic cotton encoding the *Bt cry1Ac* gene is being grown for experimental purpose. The total population of culturable bacteria and fungi in *Bt* and non-*Bt* soil samples were analyzed by dilution plating method. ANOVA analysis of the microbial population of soil samples from different locations showed no significant variation between *Bt* and non-*Bt* samples. Earthworms were not observed in the sampling locations except for one time in Kovilaptti. The level of Cry1Ac protein in the samples was determined by insect bioassays with *Helicoverpa armigera*. When the first instar of *H. armigera* were fed with the artificial diet containing the soil samples, no mortality was seen after 4 days of incubation. About 85 to 100% of larvae have reached the third instar stage after four days of incubation. Together, these findings demonstrate that the level of Cry1Ac protein present in the soil where *Bt* cotton is grown, is extremely low and does not have any adverse effect on the microbial population of the soil samples studied.

Keywords: Transgenic cotton, *Bacillus thuringiensis*, Cry1Ac protein, microflora, insect bioassay, *Helicoverpa armigera*

Introduction

Bacillus thuringiensis forms crystalline inclusions during sporulation, which contain one or more insecticidal delta-endotoxins or Cry proteins. Expression of these proteins in transgenic plants will provide resistance to various insect pests. Several crops including cotton have been genetically modified to express insecticidal proteins of *Bt*. This kind of improved delivery system of *Bt* toxin through transgenic plants has great potential because when incorporated into plants, *Bt* proteins are made much more persistent and effective against insects that feed at sites difficult or impossible to reach with sprays. It also reduces reliance on traditional chemical insecticides in insect control programs (Hoffmann et al. 1992, Roush 1994). In addition, because Cry proteins show a great deal of host specificity, with each protein being toxic to only one or a few insect species, the transgenic crop plants producing these proteins have advantages over broad-spectrum pesticides in facilitating integration of other environmentally –benign pest control strategies, such as biological control into integrated pest management programs (Bolin et al. 1996, Orr and Landis 1997, Schuler et al. 1999).

Despite the considerable advantages of *Bt* transgenic crops, both to the environment and to farmworker safety, concern is widespread that the gains will be short-lived because of a). evolution of resistance in the target pests, b). possible impacts on non-target soil organisms that are of ecological and / or economic interest. One important aspect to consider in evaluating these concerns is the possible risk of accumulation and persistence of the plant produced *Bt* proteins in soils where the crops are repeatedly grown and plant residues such as roots are plowed back into the soil. Laboratory studies (Crecchio and Stotzky, 1998, Tapp et al. 1994, Tapp and Stotzky, 1995) have shown that insecticidal toxin produced by *B. thuringiensis* subsp. *kurstaki* remains active in the soil, where it binds rapidly and tightly to clays and humic acids, and that the bound protein retains its insecticidal properties. Crecchio and Stotzky (1998) also showed that, under laboratory conditions, the *Bt* protein which had bound to soil particles degraded more slowly than free protein.

Soil sampling

Soil samples were collected periodically from the above-stated five locations and transported to the lab on ice. The first soil sampling was done at 30 days after sowing and subsequent collections were made at six weeks interval until harvest. At the end of the growing season two post season samples, at one-month interval are also scheduled in this study. For sampling purpose the area around plant selected was divided into rhizosphere and non-rhizosphere zones as shown in Fig.1

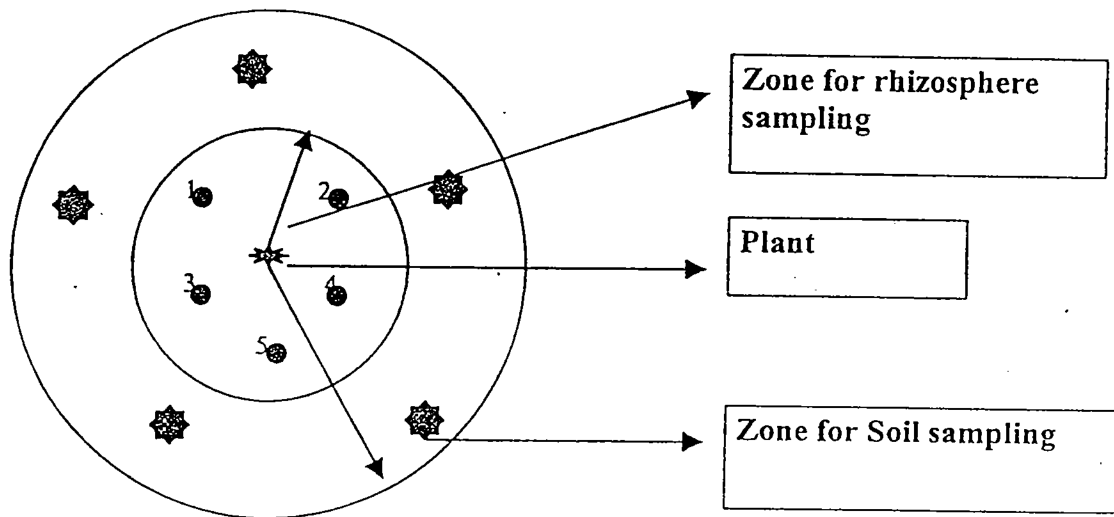

Fig.1. Zones for sample collection.

The rhizosphere zone includes 0-1 ft area around the plant and the non-rhizosphere zone represents 1-2 ft area. To get one sample from rhizosphere, five core samples, each about 5- 6 inches deep and 3 inches in diameter, were randomly taken in the rhizosphere zone and mixed thoroughly. From this 100 g of soil was drawn as a representative rhizosphere sample. Similarly a bulk sample was collected from the outer ring of 1- 2 ft area to represent the non – rhizosphere soil sample. From each location and at every sampling time point, a total of four rhizosphere and four non-rhizosphere samples were collected each from the *Bt* and non-*Bt* plots.

Determination of total bacterial and fungal populations. To determine the total bacterial and fungal populationsl, suspensions from rhizosphere / non-rhizosphere soil

samples were made in sterile distilled water by suspending 10 gms of sample in 90 ml water and shaking vigorously for 20 minutes on a Gyrorotary shaker at 250 rpm. These primary soil suspensions were serially diluted further and appropriate dilutions were plated on King's B (KB) agar medium (King et al., 1954) and potato dextrose agar (PDA) medium to study the population of bacteria and fungi respectively. Incubations were made at 26 – 28 °C for 3 and 7 days for bacteria and fungi respectively. Total number of bacterial and fungal colonies were counted after the incubation period. The differences in the total bacterial and fungal population levels among *Bt* and non *Bt* treatments were determined in SAS with Analysis of variance (ANOVA).

Analysis of soil invertebrates. Earthworm populations were monitored in the *Bt* and non-*Bt* plots at the time of soil sample collection in all the five sampling locations.

Level of Bt protein in soil:

The level of *Bt* protein in soil samples was be determined by insect bioassays with *Helicoverpa armigera* . To assay for Cry1Ac protein, soil samples were incorporated into the artificial diet and then presented to first instar of *H. armigera*. Ten grams soil from each sample was thoroughly slurried with 20 ml of sterile distilled water, in a 100 ml centrifuge tube on a vortex mixer. Five ml of this slurry was then mixed with agar – based liquid diet to bring the total volume to 25 ml. The soil-diet mixture for each sample was added to five plastic vials at the rate of five ml per cell. After the soil-diet mixture was solidified and cooled, four first instar *H. armigera* were introduced into each vial. Each treatment had five replications and a total of 20 first instar larvae. The vials were incubated at 26° C and 50 to 60 % relative humidity for four days. Following four days of incubation, survival and stages of growth of *H. armigera* were recorded. To serve as a reference standard, artificial diet mixture with different levels of purified Cry1Ac protein (0 to 800 ng per ml of the diet) were assayed with *H. armigera* as described previously.

Results

Soil samples. A total of 18 soil samples have been collected so far from the *Bt* and non-*Bt* plots at different time points. This includes four samples from Barwah, three from Davangere, four from Kallakal, two from Kovilpatti and five from Yeotmal. Soil microflora analysis and insect bioassays to determine the level of bioactive protein were completed for all the 18 samples.

Soil Microflora. For soil microflora analysis total number of bacterial and fungal colonies were counted and analysis of variance was done to determine the variance between soils from *Bt* and non-*Bt* treatments. No significant variations were observed in the culturable bacterial and fungal population among the Bt and non-*Bt* treatments irrespective of the location and also irrespective of the different sampling time points. The results for the bacterial study are presented in Table.1a - 1e. The bacterial population consisted of about six to eight different major types of colonies and no potential shift in the population level of any type bacterium was observed in this study. The type of colonies grown from *Bt* and non-*Bt* soil samples were almost similar.

The total fungal populations for the rhizosphere and non-rhizosphere samples are presented in Fig.2 and Fig. 3. Species of *Aspergillus, Penicillium, Rhizopus and Mucor* were the dominant fungi that were found to grow. Besides these, *Trichoderma, Fusarium, Gliocladium, Cephalosporium, Cladosporium* , *Myrothecium* , and *Alternaria* are the other fungi recorded..

Analysis of earthworms and Collembola. No populations of Collembola were recorded in any of the five locations at any sampling time points. Except for the third sampling time point (114 days after sowing) at Kovilaptti, earthworms were absent in both *Bt* and non-*Bt* plots of all the five locations studied. In the Kovilpatti location, during the third sampling time point, earthworms were found in the rhizosphere zones of *Bt* and non *Bt* plants. But measurable numbers of earthworms were not found in these plots.

Insect Bioassay. To determine the level of bioactive *Bt* protein present in the soil samples, insect bioassays were done with *H. armigera*. The results are presented in Fig. 4 and 5. Results from reference standards with purified Cry1Ac protein showed

that after four days of incubation, ten of 20 first instar *H. armigera* introduced were dead and other 10 were in the first instar stage at 800 ng per ml concentration of standard protein while

10 % of the larvae were dead in 400 ng/ ml. In other concentrations (100 and 200 ng/ ml) about 90-95 % of larvae were in the first instar stage while in the controls without any Cry1Ac protein all larvae were found to be in the third instar stage.

For all test samples from five different locations, all larvae were found to be alive after the four days incubation period. The results for insect bioassays with rhizosphere and non rhizosphrere soil samples are presented in Fig. 4 and 5 respectively. The mean larvae in the third instar stage was 96 to 98 % for all the *Bt* soil samples (rhizosphere + non-rhizosphere) collected at different time points, and 98% for all the non-*Bt* samples. The survival and growth rates were found to be similar for both rhizosphere and non-rhizosphere samples. In general, no significant amount of bioactive *Bt* protein was detected in the soil samples from *Bt* plots of all the sampling locations.

Discussion

An important aspect of the risk assessment of pesticidal transgenic plants encoding *Bt* insecticidal genes is the impact on the soil ecosystem by the *Bt* protein released into soil by plant residues and/ or root exudates. We evaluated this by analyzing the total population of culturable bacteria and fungi in *Bt* and non-*Bt* soil samples, quantifying the population of earthworms and Collembola, and detecting the level of Cry1Ac protein in soils by insect bioassay with *H. armigera.*

Analysis of microbial population in *Bt* and non-*Bt* soil samples clearly shows that the microbial population, both bacteria and fungi, is not affected by the Cry1Ac protein reported to be released into soil. In all the five sampling locations no statistically significant difference in the total microbial population was observed among *Bt* and non-*Bt* soil samples (Table 1a – 1e and Fig2 and 3). These results confirm our previous research done during March – April 2000 with post harvest soil samples from the *Bt* and non-*Bt* plots (Valasubramanian, 2000) . The data of this

study also corroborate the results of Donegan et al. (1995) who found that the bacterial and fungal populations were not affected by the *Bt* proteins.

Insect bioassays with *H. armigera* indicate that the level of bioactive Cry1Ac protein in the *Bt* soil samples of all five sampling locations, is very much below the level of detection or the protein is not present in those samples. These results are in par with the non-*Bt* soil samples where growth retardation of the *H. armigera* was not observed. The absence of detectable Cry1Ac protein in most of the soil samples suggests either little or no accumulation of the protein in the soil and rapid break down by plant protease and microbes. Numerous other studies, performed under realistic conditions, have shown that the *Bt* proteins produced in *Bt* crops are rapidly degraded in soil. There was no suggestion in any case that substantial amounts of protein remained undegraded in the soil (Palm et al. 1994, 1996; Ream et al. 1992; Sims and Holden 1996; Valasubramanian 2000).

To determine the effect of the Cry1Ac protein present in soil on soil invertebrates with special reference to earthworms and Collembola, we sought to quantify their population at different time points. Data could not be collected for Collembola as no Collembola was found at any sampling time point in all the five locations. Earthworms were found in the *Bt* and non *Bt* plots of Kovilpatti only during the third sampling time point (i.e., 114 days after sowing) , but measurable numbers were not seen. Other than this, earthworms were absent in both *Bt* and non-*Bt* plots of all other locations studied. However, several published evidences indicate that *Bt* proteins pose no identifiable toxicological risk to soil inhabiting non-target soil organisms including earthworms and Collembola (MacIntosh et al. 1990, Sims 1995, Yu et al,. 1997). Sims and Martin (1997) and Yu et al. (1997) showed that both pure and plant-produced *Bt* Cry proteins had no effects on the survival and reproduction of the soil inhabiting Collembola. Similar studies by registrants to United States Environmental Protection Agency have shown that earthworms also are not susceptible to the Cry proteins.

Findings from this research work to evaluate the impact of *Bt* protein on soil microflora and non-target soil organisms including earthworms, coupled with those of the other published studies, support the argument that *Bt* proteins released into soil by

plant debris, root exudates, etc., is degraded rapidly and do not adversely affect the soil microflora and earthworms.

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Table 1a. ANOVA analysis of total bacterial population from *Bt* and non-*Bt* soil samples.

Location: Barwah

Sampling time point (Days after sowing)	Treatment	No. of observation	**Mean of total bacterial colonies (-X 10^6 colony forming units per gram soil)***	
			Rhizosphere	**Non-rhizosphere**
30	Bt	8	5.2	3.7
	Non-Bt	8	4.8	3.9
	LSD		1.8	1.1
72	Bt	8	3.2	2.4
	Non-Bt	8	3.1	2.6
	LSD		1.5	0.5
114	Bt	8	4.6	4.0
	Non-Bt	8	4.3	4.2
	LSD		1.7	0.97
156	Bt	8	5.5	3.9
	Non-Bt	8	5.0	3.8
	LSD		0.9	0.96

* Mean from four different samples
LSD : Least Significant difference

Table 1b. ANOVA analysis of total bacterial population from *Bt* and non-*Bt* soil samples.

Location: Davangere

Sampling time point (Days after sowing)	Treatment	No. of observation	Mean of total bacterial colonies (-X 10^6 colony forming units per gram soil)*	
			Rhizosphere	Non-rhizosphere
30	Bt	8	3.7	3.5
	Non-Bt	8	3.0	3.3
	LSD		1.6	2.3
72	Bt	8	4.5	4.7
	Non-Bt	8	4.2	4.2
	LSD		1.6	1.3
114	Bt	8	5.1	5.7
	Non-Bt	8	4.4	5.2
	LSD		1.0	1.3

* Mean from four different samples
LSD : Least Significant difference

Table 1c. ANOVA analysis of total bacterial population from *Bt* and non-*Bt* soil samples.

Location: Kallakal

Sampling time point (Days after sowing)	Treatment	No. of observation	Mean of total bacterial colonies (-X 10 ^{6} colony forming units per gram soil)*	
			Rhizosphere	Non-rhizosphere
30	Bt	8	3.4	3.1
	Non-Bt	8	3.1	3.4
	LSD		0.52	1.5
72	Bt	8	4.8	4.2
	Non-Bt	8	3.9	4.0
	LSD		1.3	1.6
114	Bt	8	5.0	3.7
	Non-Bt	8	4.7	3.7
	LSD		2.0	1.9
156	Bt	8	5.4	4.7
	Non-Bt	8	4.2	4.8
	LSD		1.3	0.85

* Mean from four different samples
LSD : Least Significant difference

Table 1d. ANOVA analysis of total bacterial population from *Bt* and non-*Bt* soil samples.

Location: Kovilpatti

Sampling time point (Days after sowing)	Treatment	No. of observation	Mean of total bacterial colonies (-X 10 ^{6} colony forming units per gram soil)*	
			Rhizosphere	Non-rhizosphere
30	Bt	8	5.3	6.4
	Non-Bt	8	5.2	5.0
	LSD		1.7	4.7
72	Bt	8	4.3	4.3
	Non-Bt	8	3.8	3.5
	LSD		3.0	1.4

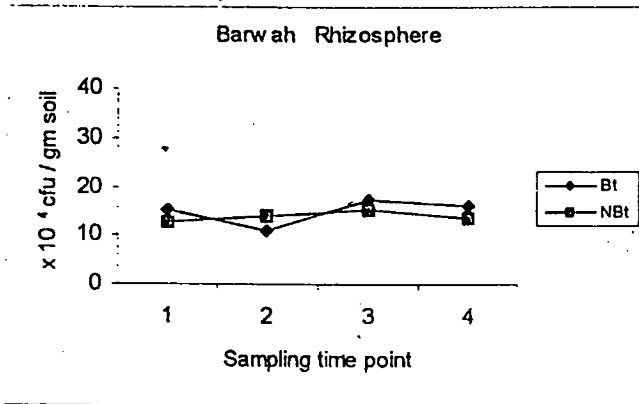
* Mean from four different samples
LSD : Least Significant difference

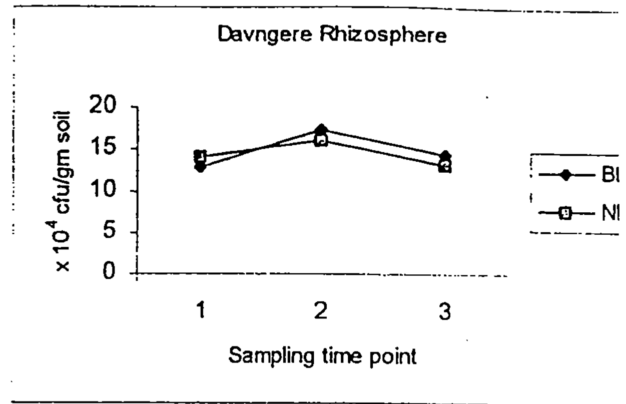
Table 1e. ANOVA analysis of total bacterial population from *Bt* and non-*Bt* soil samples.

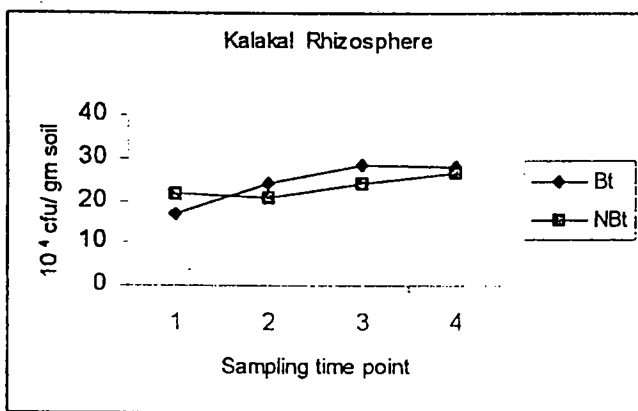
Location: Yeotmal

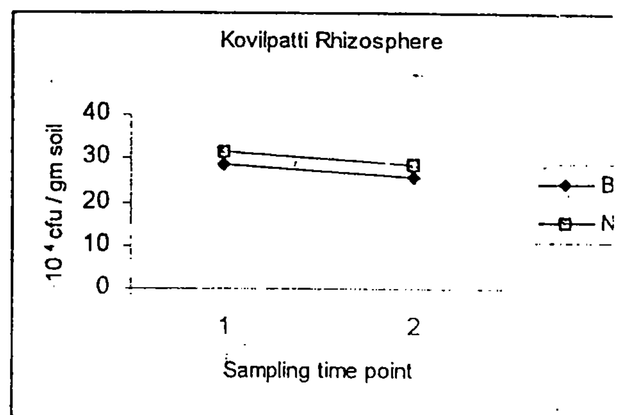
Sampling time point (Days after sowing)	Treatment	No. of observation	Mean of total bacterial colonies (-X 10^6 colony forming units per gram soil)*	
			Rhizosphere	Non-rhizosphere
30	Bt	8	2.6	3.3
	Non-Bt	8	2.8	3.5
	LSD		1.5	1.2
72	Bt	8	4.7	4.1
	Non-Bt	8	4.9	4.1
	LSD		2.3	2.6
114	Bt	8	4.4	3.9
	Non-Bt	8	4.7	3.4
	LSD		1.5	1.1
156	Bt	8	3.1	4.4
	Non-Bt	8	3.9	3.1
	LSD		1.1	1.9
208	Bt	8	5.5	4.9
	Non-Bt	8	5.0	4.8
	LSD		1.1	1.1

* Mean from four different samples
LSD : Least Significant difference









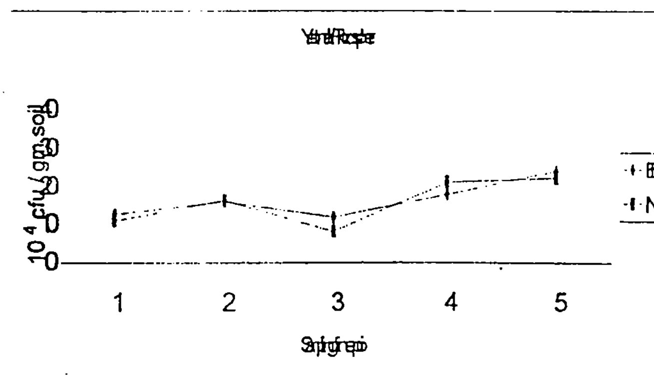
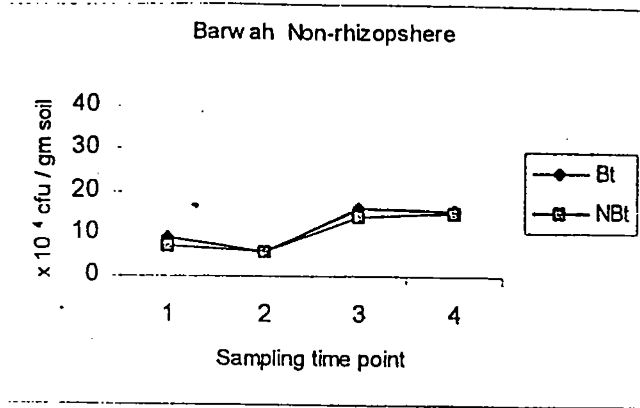
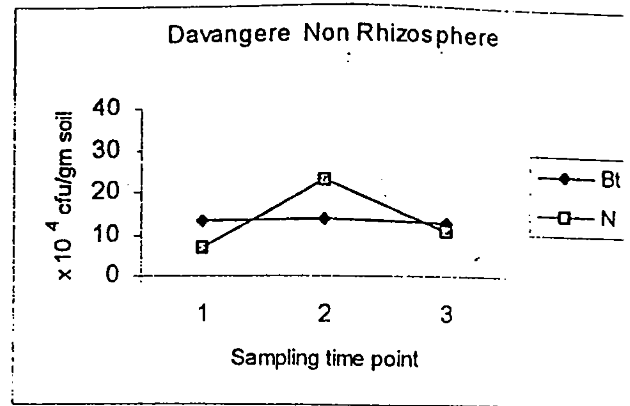
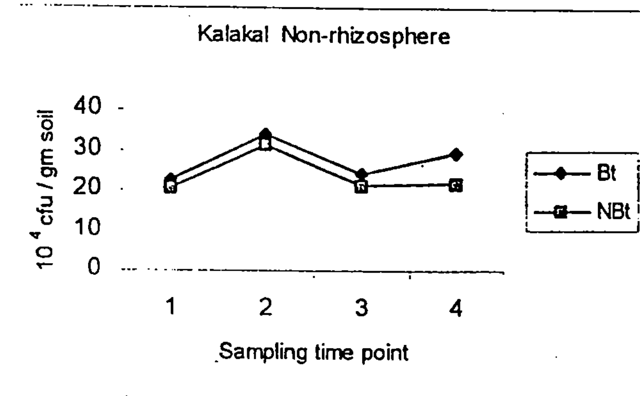
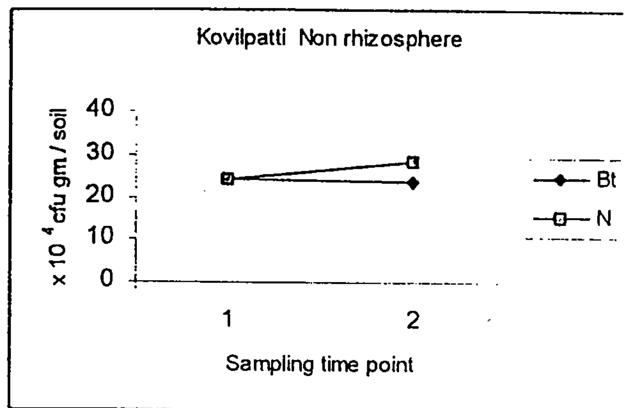


Fig 2. Effect of *Bt* protein on soil fungal population . Each value is mean of four different samples. LSD values of pooled *Bt* . *NBt* populations: Barwah – 4.9, Davangere – 3.9, Kallakal – 6.7, Kovilpatti – 10.1, Yeotmal – 5.1. Sampling time point : 1 – 30 days after sowing , 2 – 72 days after sowing, 3 – 114 days after sowing, 4 – 156 days after sowing and, 5 – 208 days after sowing.









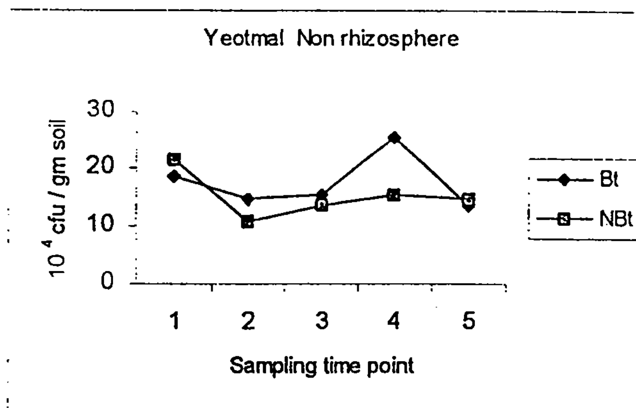


Fig 3. Effect of *Bt* protein on soil fungal population . Each value is mean of four different samples. LSD values of pooled *Bt / NBt* populations: Barwah – 5.3, Davangere – 7.4, Kallakal – 7.4, Kovilpatti – 6.1, Yeotmal – 5.3 . Sampling time

Point : 1 – 30 days after sowing , 2 – 72 days after sowing, 3 – 114 days after sowing, 4 – 156 days after sowing and, 5 – 208 days after sowing.

Fig. 4 Insect bioassays to determine the level of *Bt* protein in rhizosphere soil samples

Each value is a mean of 4 different samples. Results were recorded after 4 days of incubation.

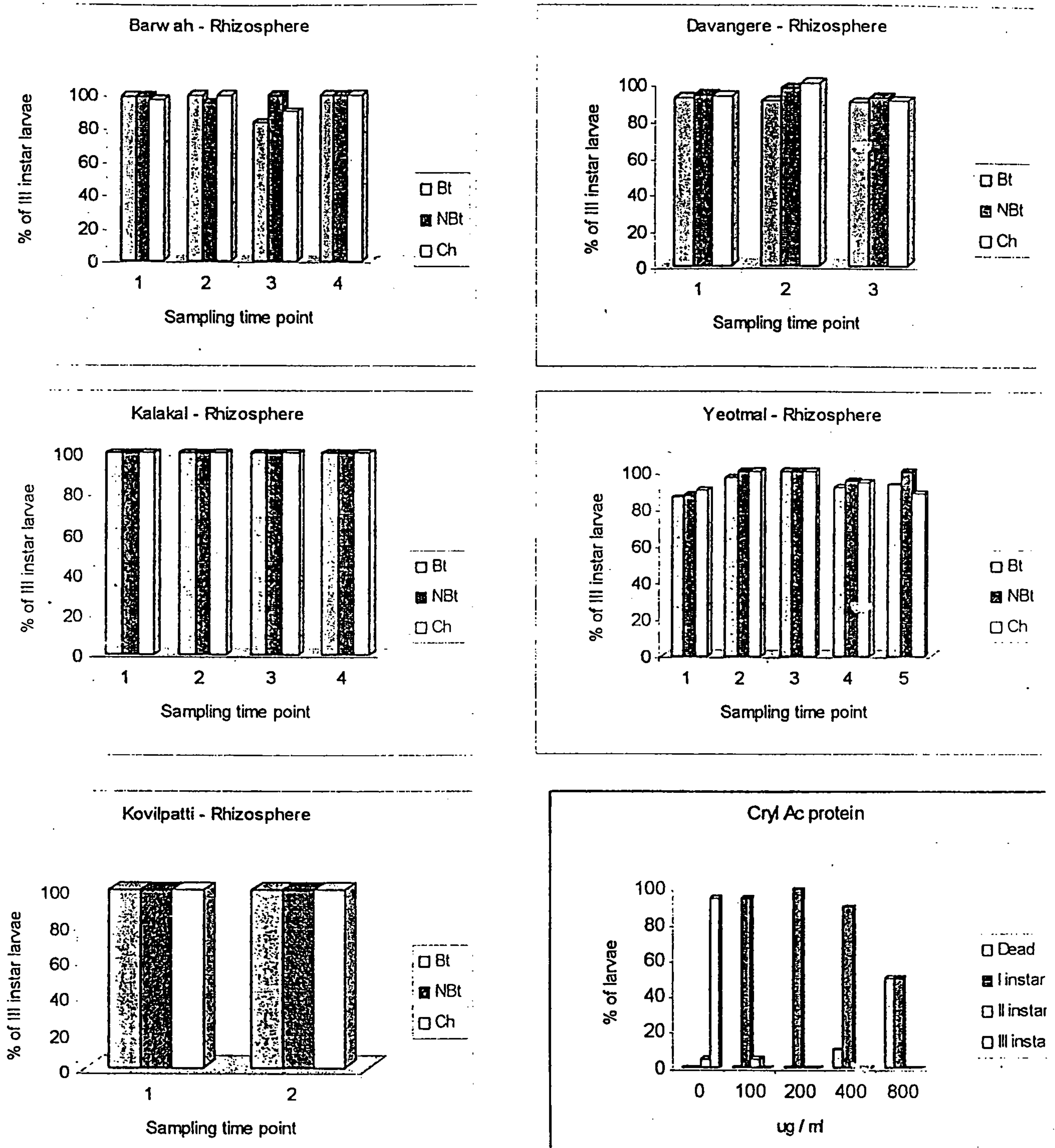
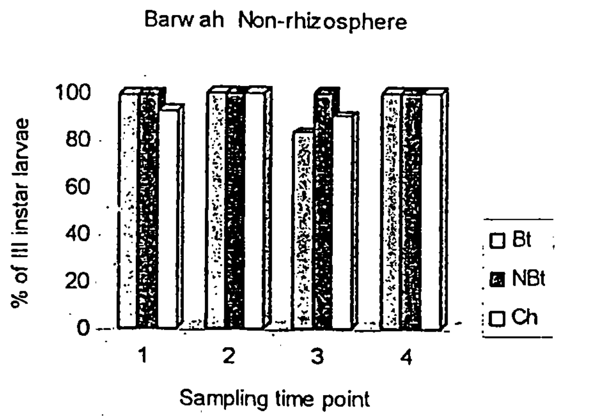
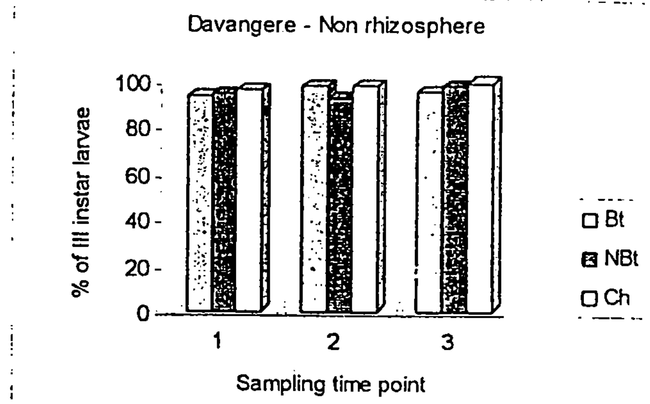
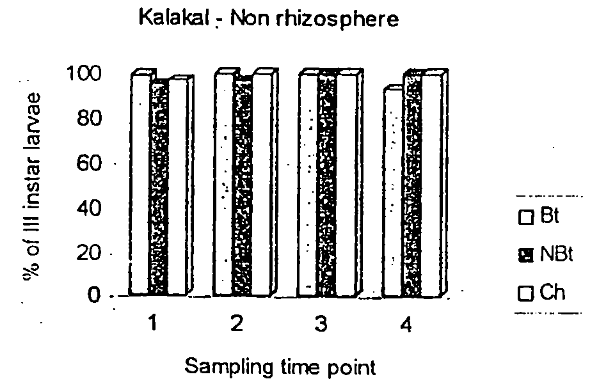
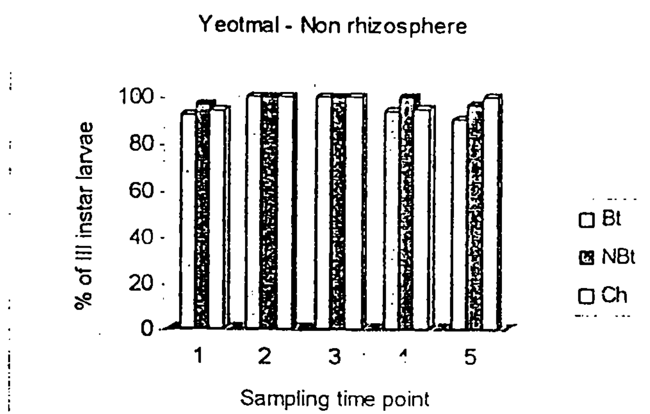


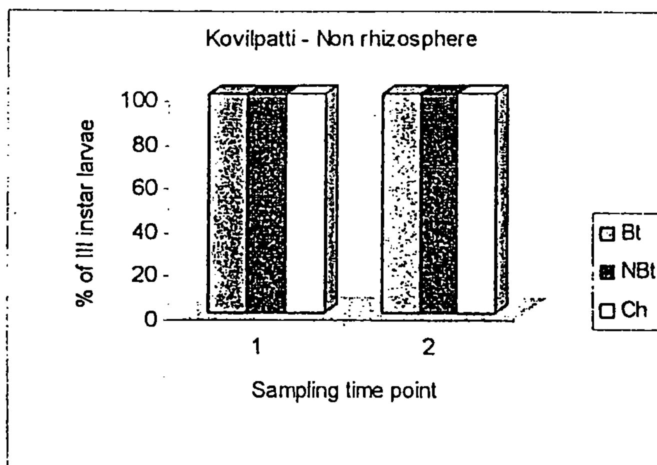
Fig 5. Insect bioassays to determine the level of *Bt* protein in non-rhizosphere soil samples collected from *Bt* and non-*Bt* experimental plots. Each value is a mean of four different samples. Results were recorded 4 days after incubation.











Pollen Flow Study of Bt Cotton in One Location

Evaluation of cross pollination in between Bt and non-Bt cotton in the presence of honey bee pollination agents

Protocol – 3 Report

Submitted to MEC & RCGM
March 2001

MAHARASHTRA HYBRID SEEDS COMPANY. LTD.
Resham Bhavan, 4th Floor, 78 Veer Nariman Road, Mumbai 400 020, India

Title of study: Pollen Flow Study of Bt Cotton in One Location

Objective: To evaluate cross pollination in between Bt and non-Bt cotton in the presence of Honey-bee pollination agents

Location of study: Maharashtra Hybrid Seeds Co. Ltd., Jamwadi Farm, Jalna (Survey No. 198, Taluka Jalna, District Jalna, Maharashtra)

Duration of study: 10 August, 2000 to 16 March, 2001.

Methods: The approved protocol for this study is given in Annexure I. Transgenic Bt cotton, homozygous for the Bt locus, was planted in a central plot measuring 20m X 20m, surrounded by non-transgenic cotton in 5 m X 5 m blocks in all four directions, starting from 1 meter to 55 meters from the central plot. Each of these blocks were composed of 5 rows, 1 meter apart. The Bt cotton line planted in the central plot had a visible genetic marker, i.e., okra leaf, while the non-transgenic pollen trap plants were of normal leaf phenotype. This arrangement was incorporated in the experiment in order to be able to score cross-pollination events in a grow-out-test of the seeds from the pollen trap plants. A 50 meter isolation distance was maintained for this plot.

To facilitate cross pollination, four honey bee hives containing active colonies were placed in the four corners of the central transgenic cotton plot. Photographs of the plot taken from all four directions are given in Figures 1 to 4.

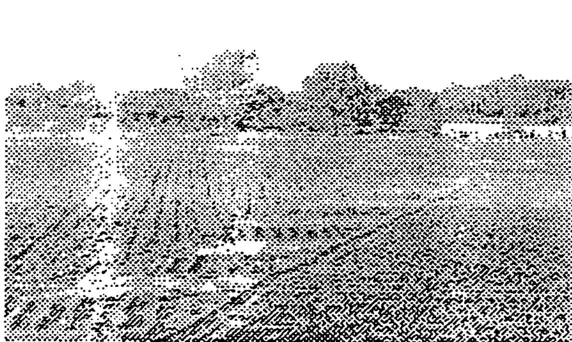


Figure 1: View towards North side of the pollen flow experimental field

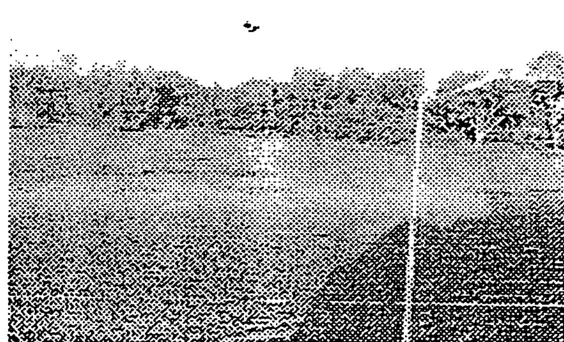


Figure 2: View towards East side of the pollen flow experimental field

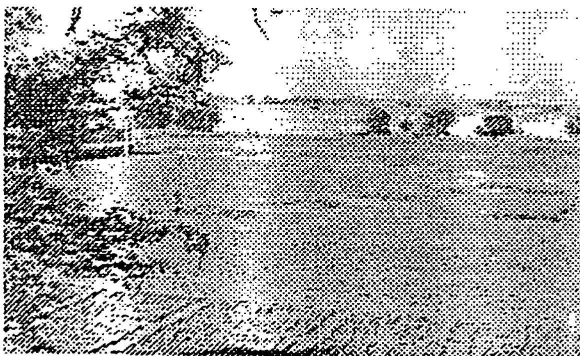


Figure 3: View towards South side of the pollen flow experimental field

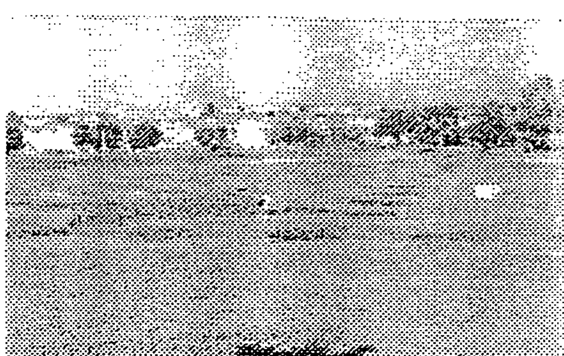


Figure 4: View towards West side of the pollen flow experimental field

Cont'd ... 2

Honey bee activity was periodically monitored to ensure the presence of adequate numbers of the cross-pollinator insect. Figures 5 and 6 show honey bees visiting the transgenic plot and the non-transgenic plot respectively.

Figure 5: Honeybee visiting Bt Cotton (Okra Leaf) in the central plot

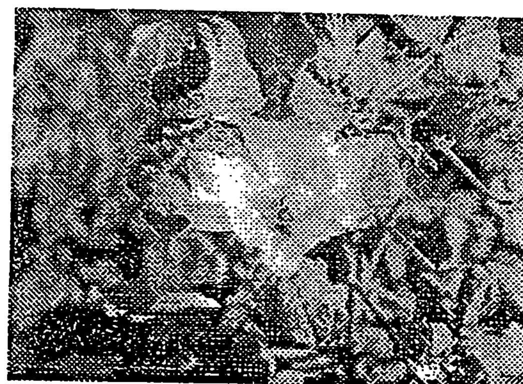


Figure 6: Honeybee visiting normal leaf plant in a pollen trap block

Weather data was periodically recorded at the experimental site, as these parameters might influence the degree and extent of pollen flow. Monthly averages of weather data are given in Annexure II.

Seed Sample Collection: Seeds were collected seperately from each row of the first block. i.e., 1 meter to 5 meters distance from the Bt cotton plot and as single bulks from each of the distal (second to the eleventh) blocks. Three random samples were drawn from each of the above collected seed lots. Thus there were 60 samples (15 per block X 4 directions) for the first set of blocks and another 120 samples (3 per block X 10 blocks X 4 directions) for the second to eleventh set of blocks. These sample lots were subjected to a grow-out-test for scoring semi-okra leaf type plants. Also a polymerase chain reaction (PCR) analysis was done on these sample lots, to detect presence of the Bt (Cry1Ac) gene in the progeny of the pollen trap plants.

Grow-out test: Fifty seeds per sample were planted in a row and the seedlings were allowed to grow for 42 days. The number of semi-okra plants that were seen in each row were counted. This gave an indication of cross pollination from the okra-leaf transgenic Bt cotton plot to the normal-leaf non-transgenic pollen trap blocks.

The grow out test (GOT) was performed in the Jamwadi farm. Each sample population was planted in a single row. Thus a total of 180 rows were planted with 50 seedlings in each. Figure 7 is an illustration of a semi-okra phenotype plant, representing a Bt cotton X non-Bt cotton cross pollination event in a GOT row. A normal leaf plant of the same row is included in the frame for comparison.

Cont'd 3

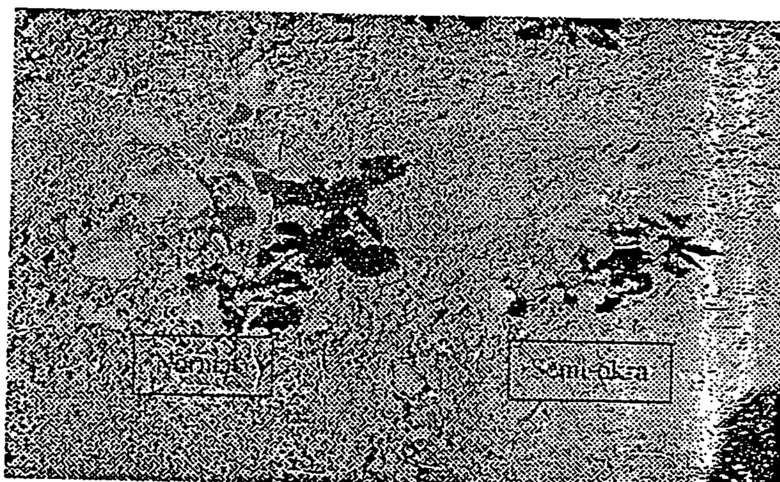


Figure 7: Semi-okra leaf phenotype appearing in the GOT

PCR analysis: 20 seeds per sample were germinated and the pooled DNA from the same was extracted. Polymerase chain reaction was performed with primers specific to the Bt Cry1Ac gene. The nucleotide sequence of these were as follows:
5' - CTG CTG AGC GAG TTC GTC CC - 3' (forward primer)
5' - GGT CTC CAC CAG TGA ATC CTG G - 3' (reverse primer)
As a positive control, a known Bt positive cotton plant DNA mixed 1:20 with non-transgenic cotton plant DNA, was used in PCR with the same primers as the other samples. The PCR products were run in a 1% agarose gel stained with ethidium bromide and the images of the gels were recorded in a computerised gel-image documentation system. The expected amplified fragment of the Bt Cry1Ac gene was 1.35 Kbp in length. The negative control in the reaction was non-transgenic cotton DNA.

Results: The result of the grow-out-test is summarised in Table No. 1. The result of the PCR analysis are shown as gel-image documents in Annexure III (Sheets 1 to 6) and the same is summarised in Table No. 2. Events of cross pollination were detected upto the third block, i.e., a distance of 15 meters, both by the grow-out-test and pooled sample PCR. The data of the latter by and large corroborated those of the former, giving credence to the sampling procedure adopted in this study.

The extent of cross pollination observed, from the Bt-cotton plot to the non-Bt cotton, ranged from averaged 0 to 3.33% in any given row within the first 5 meters of the transgenic plot. For individual samples of 50 seeds, the range varied from 0 to as high as 8% (the highest figure being for a particular sample points at 2 meters). The cross-pollination frequencies for the 6 to 10 meter block ranged from 0 to 2 %, for a given block, while the average for all four blocks at this distance interval was 1.16%. In the third block, i.e., 11 to 15

Cont'd 4

meters, the average cross pollination recorded for this distance interval was 0.5%, the range being from 0 to 1.33 % for individual blocks.

Conclusions and Discussion : Both the grow-out test and the PCR test on the progeny samples drawn randomly from the pollen-trap blocks, indicate a detectable level of pollen flow from the central transgenic plot upto Block 3 of the experiment.. While there are differences in the observed cross-fertilisation events among some of the direction-wise blocks, when comparing the grow-out test and the PCR test data, the distance limit of detectable dispersal is common in both these cases. These differences may be attributed to the chance presence of individuals arising out of cross-fertilisation, in the various samples. The sample size of 50 seeds X 3 samples per distance point scored in the grow-out test provides a limit of detection approximately 0.66% for a particular distance point in a given direction. This figure is arived at by applying the formula: 1 event out of 50 X 3 samples, for a given distance point in a particular direction sampled. All the four directions taken together lowers the limit of detection to 1 event in 600 individuals sampled, i.e., approximately 0.16%.

Based on this rationale, it may be concluded that in this particular experiment pollen flow from the transgenic Bt cotton source could be detected upto a distance of 15 meters. Beyond this distance, instances of pollen flow were not observed, subject to the 1 in 150 samples (0.66%) limit of detection for a given direction, or 1 in 600 samples (0.16%) in the case of all four directions taken together. Within the first 15 meters from the transgenic block, appreciable cross pollination took place in the individual rows of the first block, i.e., up to 5 meters, where-in a range of 0 to 3.33% was observed in individual rows. The overall average out crossing noticed for all the four blocks taken together in the 1 meter to 5 meters distance interval was 1.16%.

In the second block, i.e., between 6 and 10 meters, a reduced range of 0 to 2% cross-pollination frequency was observed in individual blocks. The over all average out crossing noticed for all the four blocks taken together in the 6 to 10 meters distance interval was 1.16%. In the third block, the range of observed cross pollination frequencies was nil to 1.33% in was observed in individual blocks, while the over all average for all the four blocks taken together in the 11 meter to 15 meters distance interval was 0.5%.

The difference in the range of pollen flow recorded in the 1997-98 experiment conducted in the same site and the present study may be attributed to different climatic/weather/environmental conditions causing variable pollinator activity and physical properties of the pollen itself. Also, the open field layout of discreet blocks of the pollen trap, vis-à-vis unbroken concentric squares at close proximity to each other, may have caused more detectable pollen flow in the current experiment. Noted in the perspective of a maximum of 2% genetic impurity permitted in varietal cotton seeds, the low figures of cross pollination

Cont'd 5

frequencies observed at a distance of 6 to 15 m are within the tolerance limit. Beyond 15 meters, no cross pollination was noted in this experiment subject to the lower limit of detection of 0.67% for a given distance interval in a single direction from the transgenic (Bt-cotton) plot and 0.16% for a given distance interval in all four directions.

ANNEXURE I

APPROVED PROTOCOL : To assess the pollen escape in Bt - cotton in open environment by HoneyBees.

Objective : The trial will be conducted to evaluate cross-pollination in between Bt and non-Bt cotton in presence of Honeybee on pollination agent (Entamophilous).

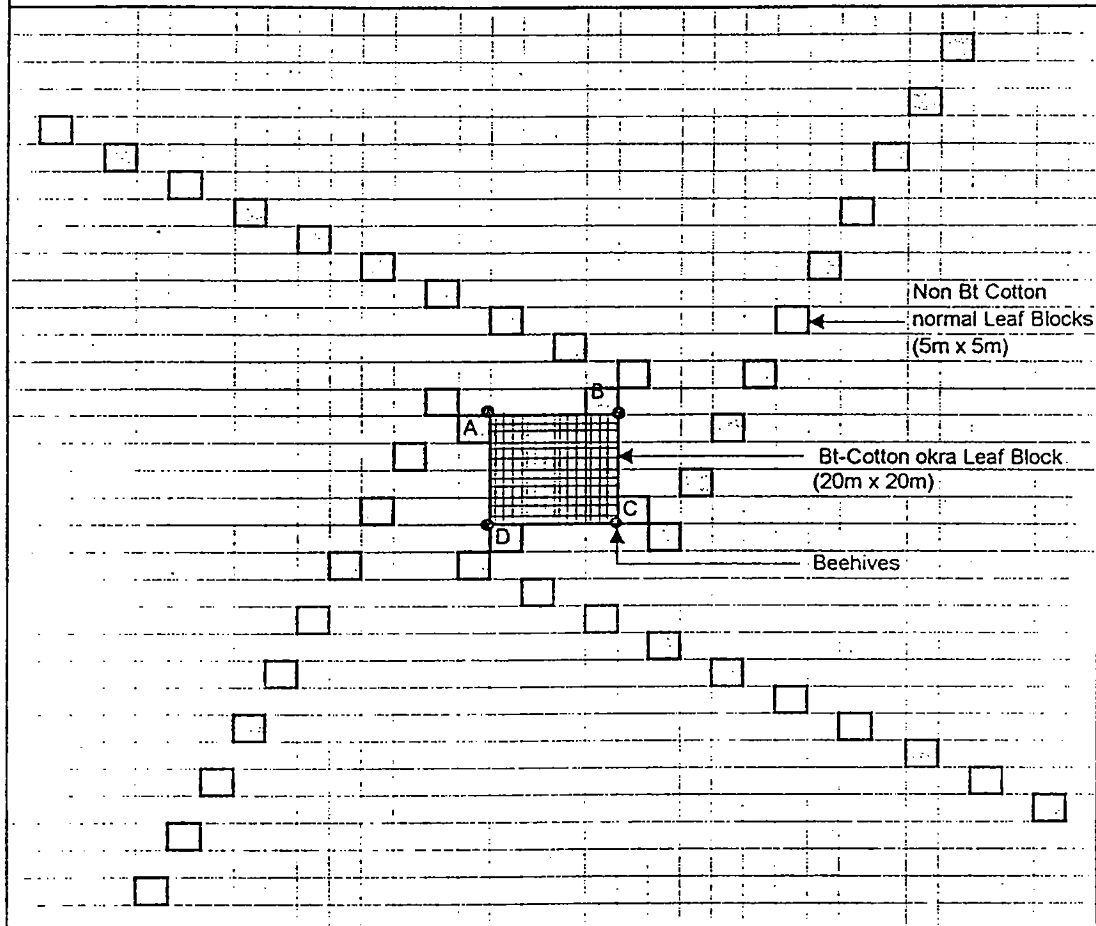
Locations : Mahyco farm located at Jalna (MS)

Materials and Methods : This experiment will be conducted at Jamwadi, District Jalna in Maharashtra. . A compact block of Bt cottons of okra leaf type in the area of 20m x 20m will be planted in the center. On four sides of this block at a distance of five meter of each 10 blocks of 5m x 5m area will be grown with non-Bt cotton of normal leaf type, in accordance with the design of the plot given below.

The first four square blocks indicated in the drawing by the letter A, B, C & D non Bt Cotton of normal leaf type shall be planted at a distance of 1 m each in the row extending upto 5 m (5 rows) in each block. To facilitate pollen dispersal four beehives shall be installed and maintained during the course of experiment. Each block other than the first four blocks adjacent to the Bt cotton block will be harvested separately, and seed sample will be drawn from the bulk of each block. Each drawn sample shall be tested for the assessment and extend of Bt contamination by PCR and grow out test. There will be another 40 blocks from which seeds would b sampled and tested. From each block minimum three seed samples be drawn and tested. Regarding the first four adjacent block seeds from each row will be collected and polled together during different times of the full harvesting season. There would be five samples from each adjacent block representing each row. Three experimental samples from the poll of each row will be drawn and the extent of Bt contamination by PCR and grow out tests will be conducted. In summary there would be 20 rows in the first four adjacent square block comprising 60 samples to be tested and from the remaining 40 square blocks there would be 120 samples which are also to be tested as above. All the experimental information shall be recorded.

A detailed schematic diagram of the approved field layout of the pollen flow experiment is given below.

FIELD LAYOUT PLAN FOR STUDY OF HONEY BEES AND POLLEN DISPERSAL



Each square block = 5m^2
Distance may be ascertained by considering each arm of each smaller square as 5m
Experimental area shown within four boundary lines

Location :
Maharashtra
Jamwadi Farm (Survey No. 198)
Taluka &District Jalna, Maharashtra

Observations: Drawn samples shall be tested for the assessment and extent of Bt-contamination by PCR and grow out test. In grow out test, presence of Semi-Okra plants will be indicative of cross pollination which will subsequently be confirmed by PCR method. The percentage of Bt. Contamination will be determined at minimum and maximum distances.

---------- End of Approved Protocol ------------

ANNEXURE II :

Weather Data at Jamwadi Farm During the Pollen Flow Experiment of Kharif- 2000-01

Date of Sow 10/08/00

Crop Durati 10/08/00 to 18/2/2001

Month	Temparature (C.)			Relative Humidity (%)			Rainfall (mm)	Wind Velocity Average(kmph)
	Maximum	Minimum	Mean	Maximum	Minimum	Mean		
August	31.50	23.90	27.70	90.20	43.10	66.60	240.20	10.05
September	31.70	22.80	27.20	92.70	34.00	63.40	0.00	7.23
October	34.10	21.10	27.60	67.50	13.10	40.30	0.00	5.1
November	30.90	17.50	24.20	78.30	17.10	47.70	0.00	4.3
December	28.40	13.60	21.00	79.60	21.20	50.40	0.00	3.85
January	29.10	16.20	22.70	83.30	24.40	53.90	0.00	Not available

ANNEXURE -III

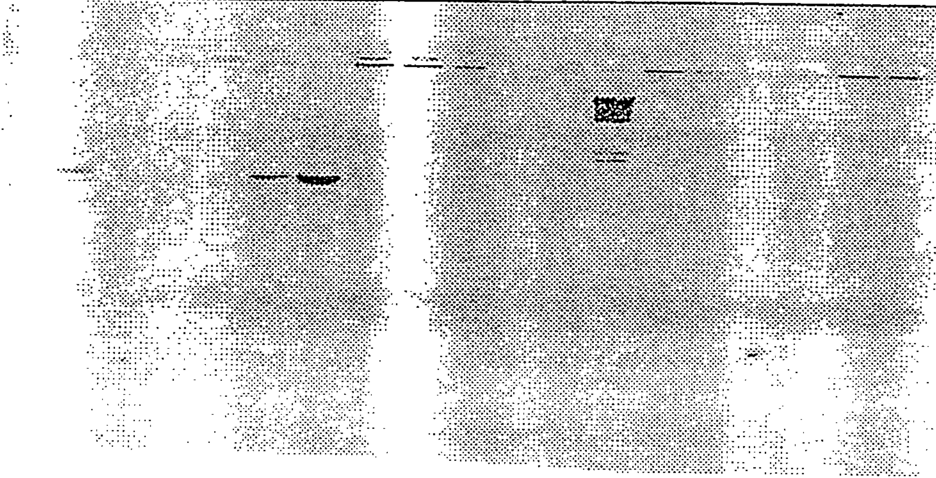
POLLEN FLOW EXPERIMENT,PCR RESULTS.

SHEET-1

CONTROL BLOCK A-1 JALNA

H2O+VE–VE 1/1 1/2 1/3 2/1 2/2 2/3 3/1 3/2 3/3 λ

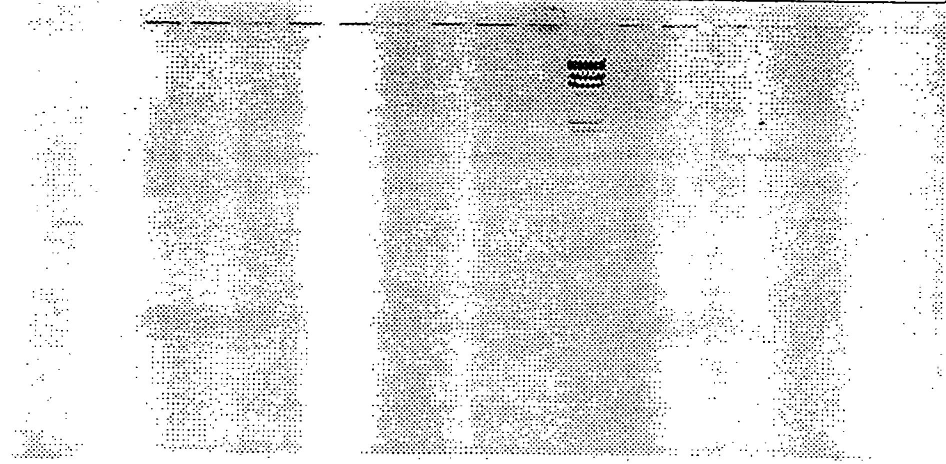
HIII 4/1 4/2 4/3 5/1 5/2 5/3



CONTROL BLOCK B-1 JALNA

H2O –VE 1/1 1/2 1/3 2/1 2/2 2/3 3/1 3/2 3/3 λ

HIII 4/1 4/2 4/3 5/2 5/3



ANNEXURE -III

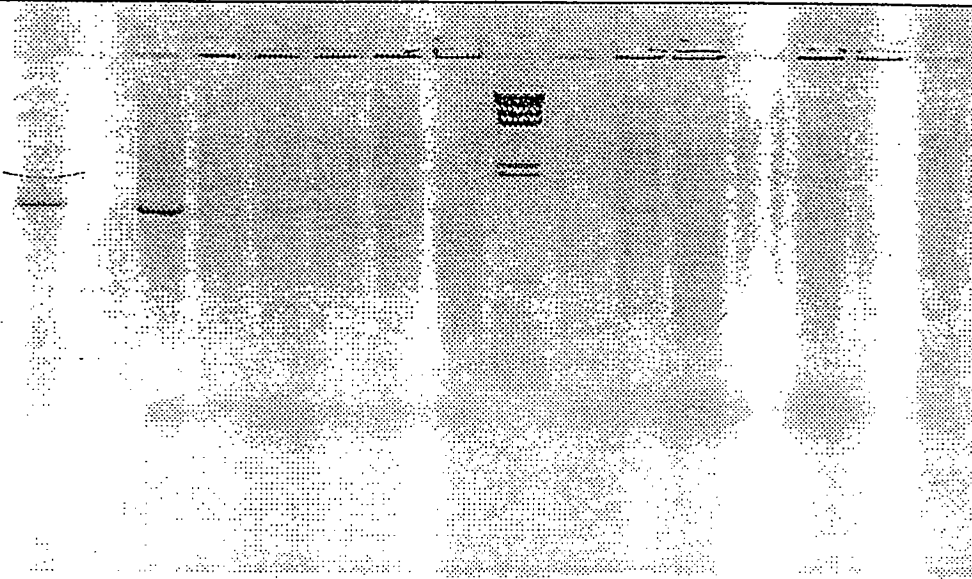
POLLEN FLOW EXPERIMENT,PCR RESULTS.

SHEET-2

CONTROL BLOCK C-1 JALNA

H2O +VE –VE 1/1 1/2 1/3 2/1 2/2 2/3 λ

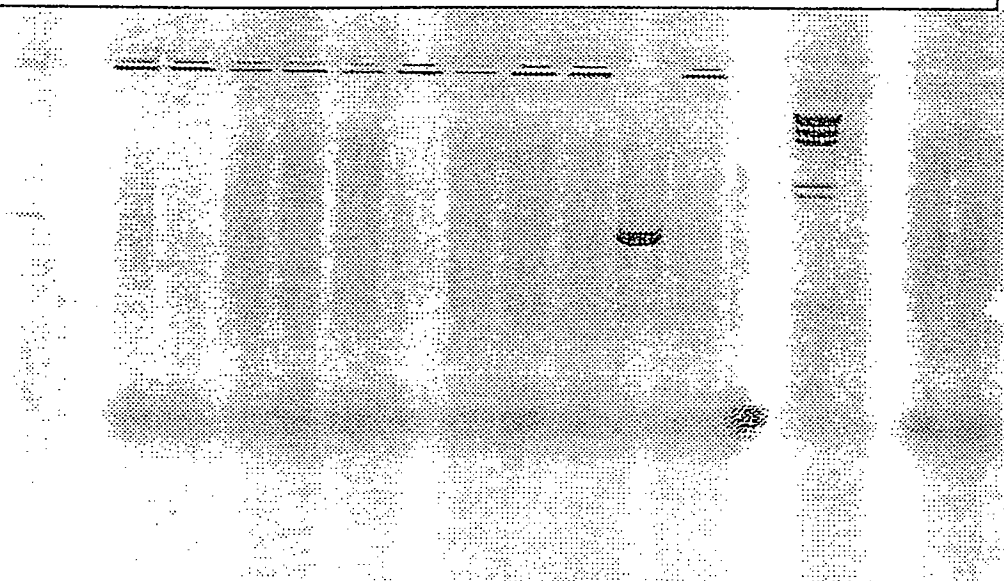
HIII 3/1 3/2 4/1 4/2 4/3 5/1 5/3



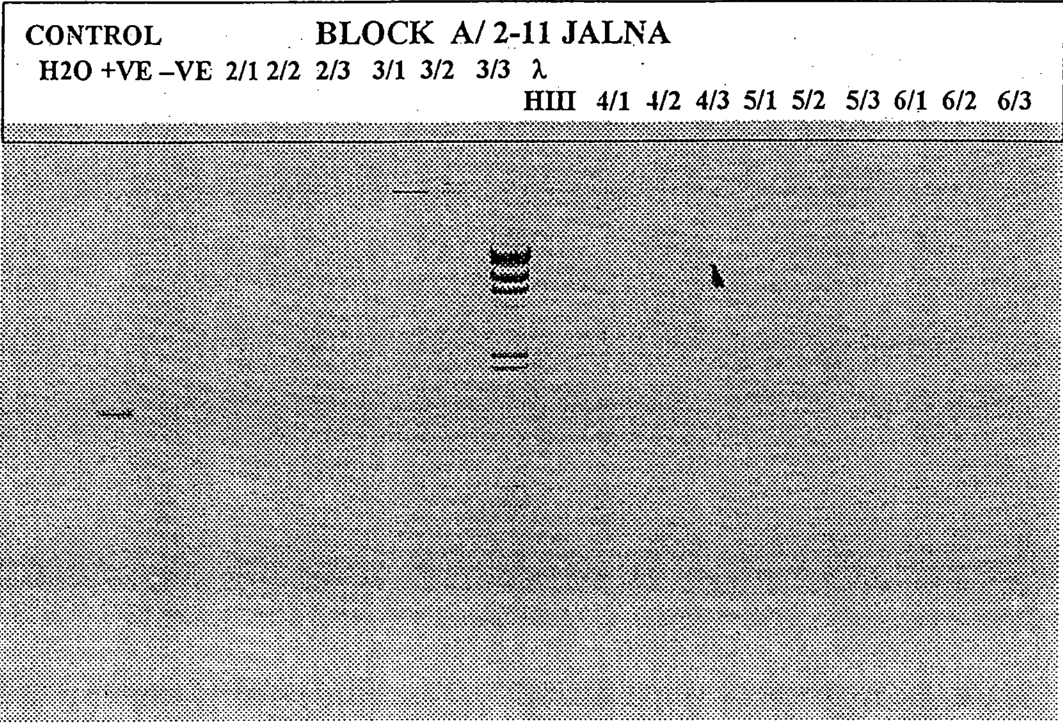
CONTROL BLOCK D -1 JALNA

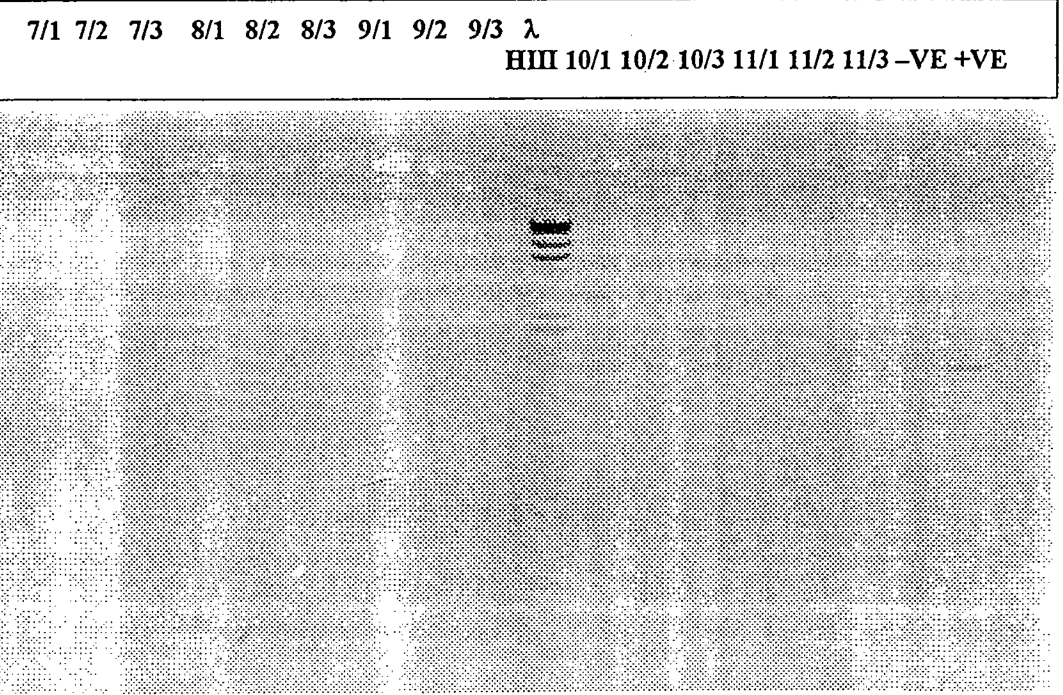
H2O +VE –VE 1/1 1/2 1/3 2/1 2/2 2/3 3/1 3/2 3/3 4/1 4/2 4/3 λ

HIII 5/1 5/2 5/3



ANNEXURE -III
POLLEN FLOW EXPERIMENT,PCR RESULTS.
SHEET-3

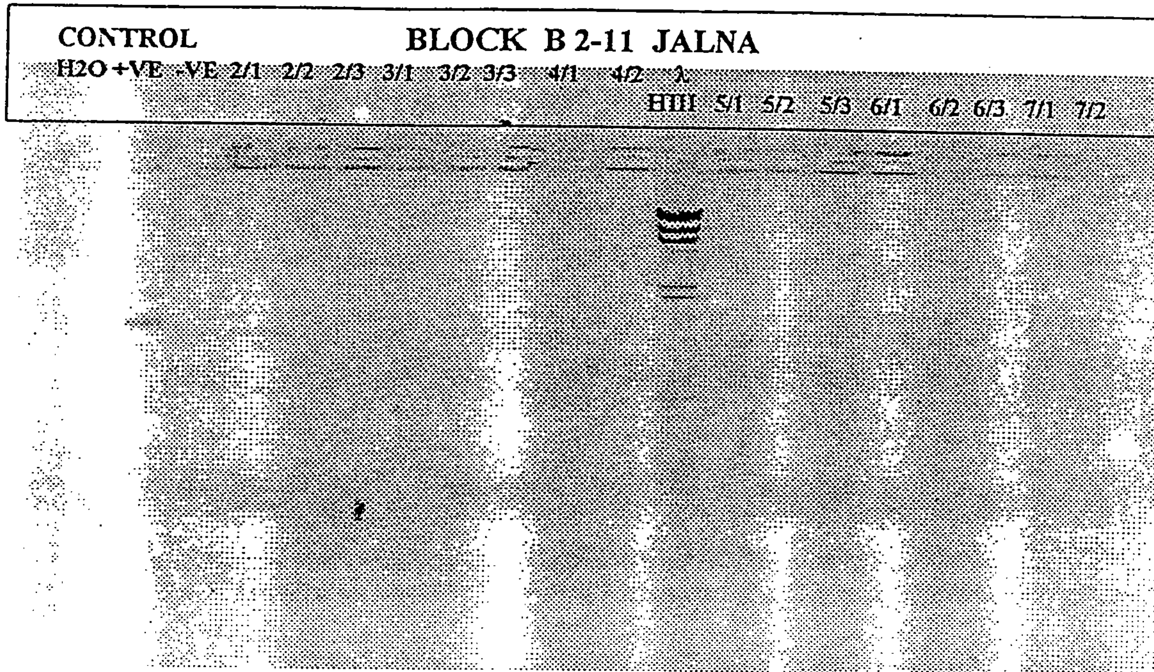


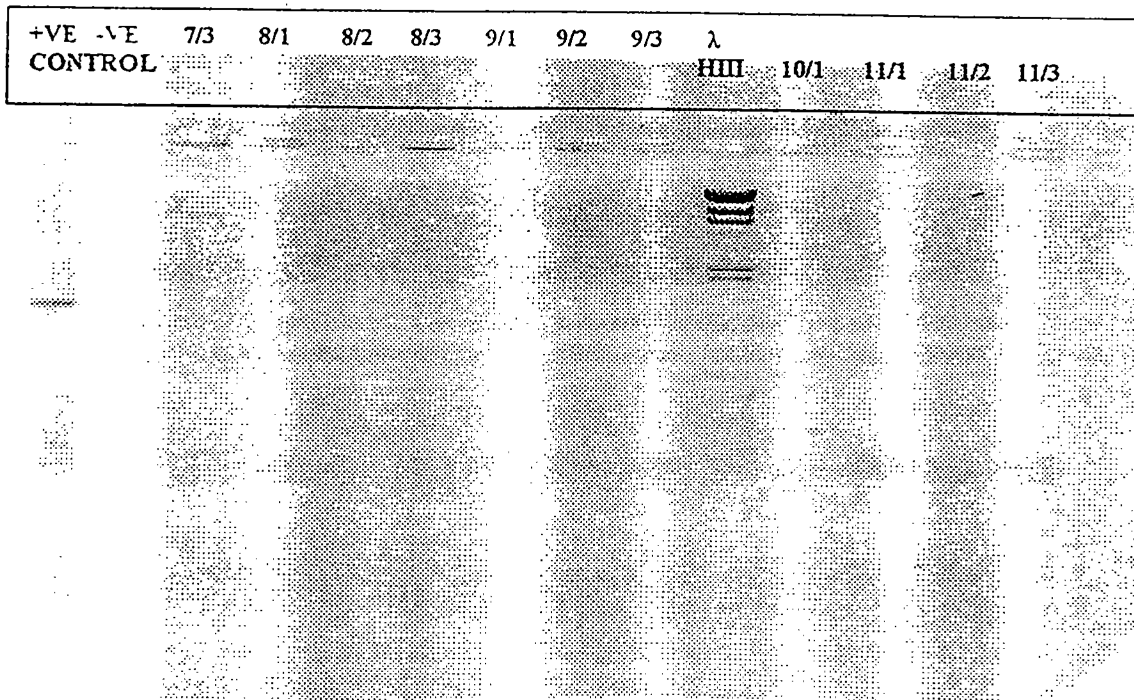


ANNEXURE -III

POLLEN FLOW EXPERIMENT,PCR RESULTS.

SHEET-4





ANNEXURE -III

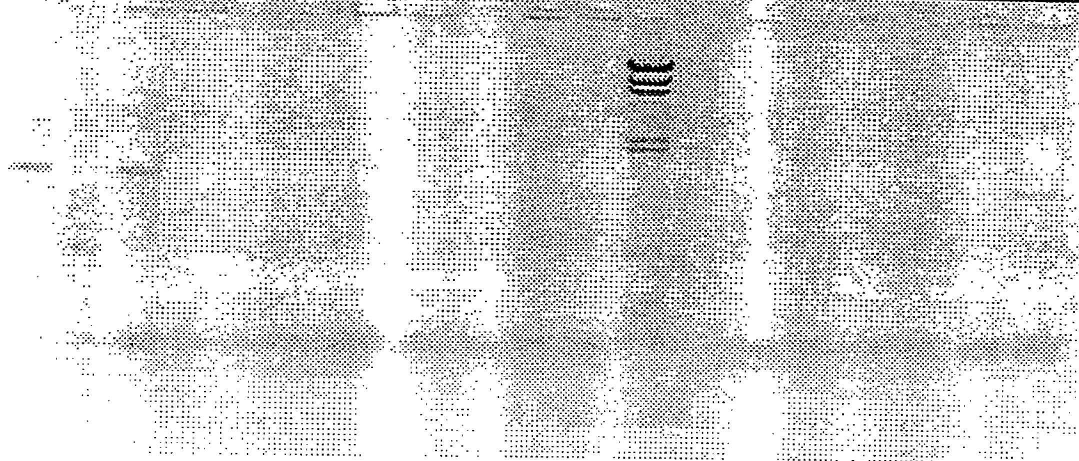
POLLEN FLOW EXPERIMENT,PCR RESULTS.

SHEET-5

CONTROL BLOCK C 2-11 JALNA

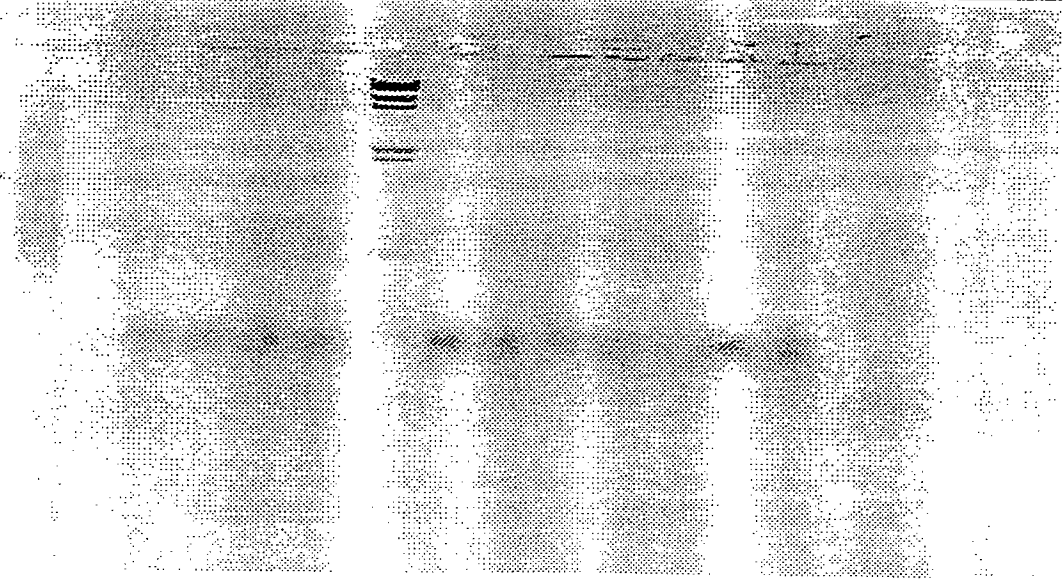
H2O +VE -VE 2/1 2/2 2/3 3/1 3/2 3/3 4/1 4/2 4/3 λ

HIII 5/1 5/2 5/3 6/1 6/2 6/3 7/1



+VE -VE 7/2 7/3 8/1 8/2 8/3 λ

CONTROL HIII 9/1 9/2 9/3 10/1 11/1 11/2 11/3



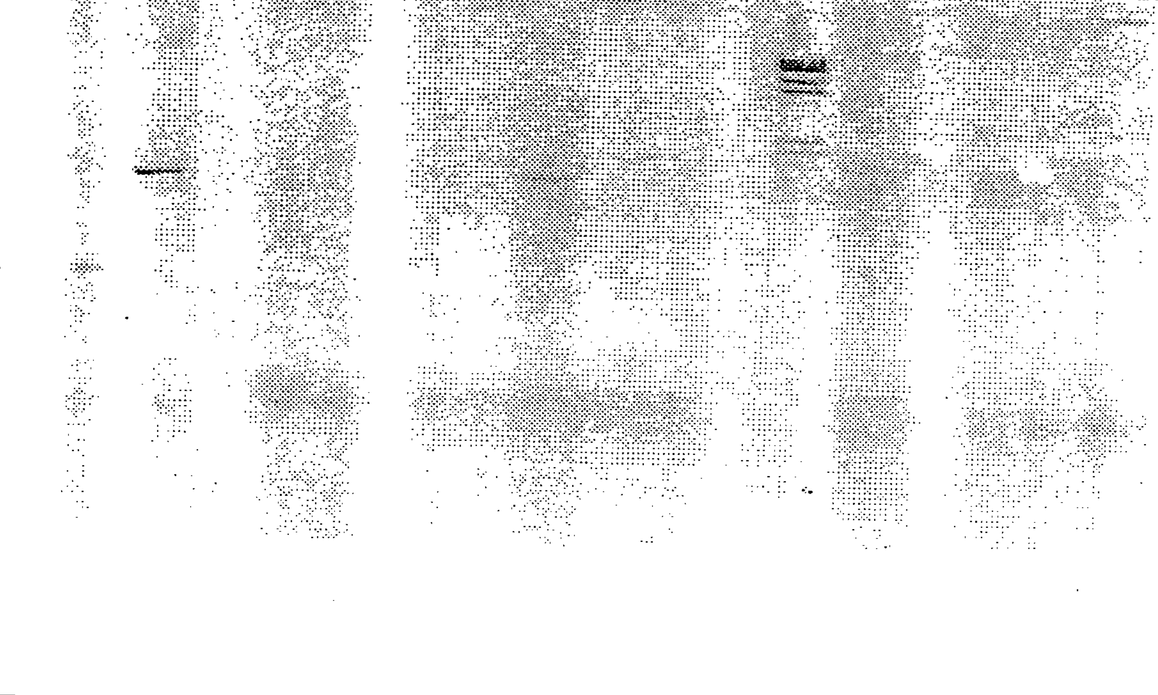
ANNEXURE -III

POLLEN FLOW EXPERIMENT,PCR RESULTS.

SHEET-6

CONTROL BLOCK D 2-11 JALNA

H2O -VE +VE 2/1 2/2 2/3 3/1 3/2 3/3 4/1 4/2 4/3 λ HIII 5/1 5/2 5/3 6/1 6/2



+VE -VE 6/3 7/1 7/2 7/3 8/1 8/2 λ HIII 8/3 9/1 9/2 9/3 10/1 10/2 10/3 11/1 11/2

CONTROL

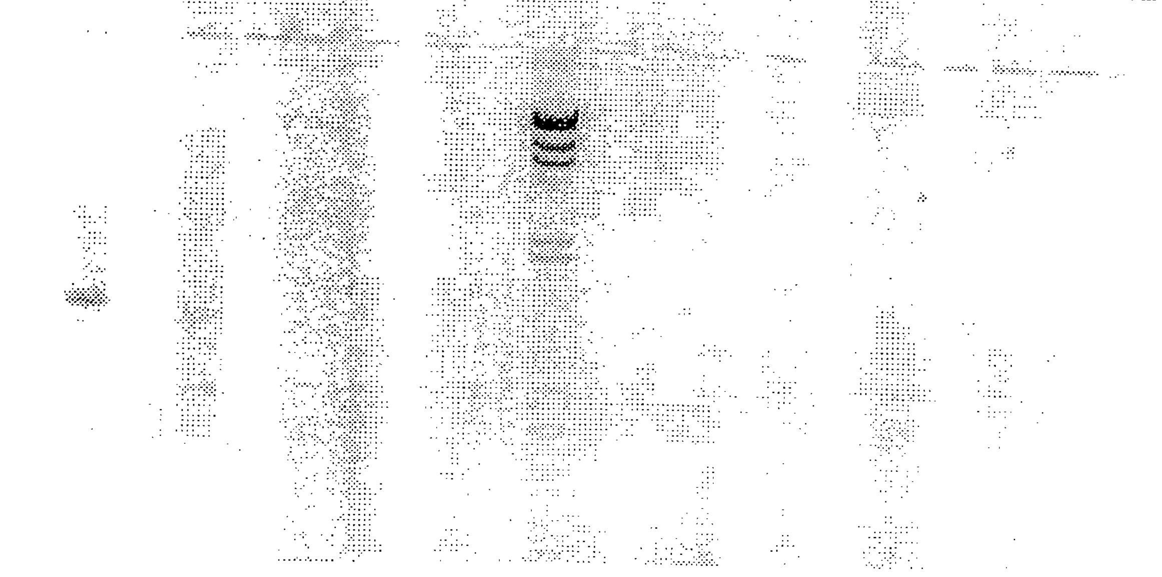


TABLE 1:

SUMMARY OF POLLEN TRAP GROW-OUT-TEST

(No. of Semi Okra individuals among the pollen trap progeny samples)

DIRECTION OF BLOCKS

		A (EAST)			B (SOUTH)			C (WEST)			D (NORTH)		
	Sample No:	1	2	3	1	2	3	1	2	3	1	2	3
Block No.1	Row No.1	1	1	0	0	0	0	2	0	2	2	1	0
	Row No.2	0	0	1	0	0	4	0	0	0	0	0	0
	Row No.3	1	1	0	2	0	1	2	0	1	0	0	1
	Row No.4	0	0	0	0	0	1	1	1	2	1	1	3
	Row No.5	0	0	0	0	1	0	0	0	0	0	0	1
Block No.2		1	1	0	0	1	1	1	0	2	0	0	0
Block No.3		0	0	1	0	0	0	0	0	0	1	0	1
Block No.4		0	0	0	0	0	0	0	0	0	0	0	0
Bolck No.5		0	0	0	0	0	0	0	0	0	0	0	0
Block No.6		0	0	0	0	0	0	0	0	0	0	0	0
Block No.7		0	0	0	0	0	0	0	0	0	0	0	0
Block No.8		0	0	0	0	0	0	0	0	0	0	0	0
Block No. 9		0	0	0	0	0	0	0	0	0	0	0	0
Block No.10		0	0	0	0	0	0	0	0	0	0	0	0
Block No.11		0	0	0	0	0	0	0	0	0	0	0	0

TABLE 2:

Summary Results of Pollen Trap Progeny PCR

DIRECTION OF BLOCKS

		A (EAST)			B (SOUTH)			C (WEST)			D (NORTH)	
	Sample No.	1	2	3	1	2	3	1	2	3	1	2
Block No.1	Row No.1	--	--	+	--	--	--	+	--	--	+	--
	Row No.2	+	--	--	+	--	--	--	--	--	--	--
	Row No.3	--	--	--	--	+	--	--	+	--	--	--
	Row No.4	--	--	--	--	--	--	--	--	--	+	--
	Row No.5	--	--	--	--	+	--	--	--	--	--	--
Block No.2		--	--	--	--	--	--	+	--	--	--	--
Block No.3		--	+	--	--	--	--	--	--	--	--	--
Block No.4		--	--	--	--	--	--	--	--	--	--	--
Bolck No.5		--	--	--	--	--	--	--	--	--	--	--
Block No.6		--	--	--	--	--	--	--	--	--	--	--
Block No.7		--	--	--	--	--	--	--	--	--	--	--
Block No.8		--	--	--	--	--	--	--	--	--	--	--
Block No.9		--	--	--	--	--	--	--	--	--	--	--
Block No.10		--	--	--	--	--	--	--	--	--	--	--
Block No.11		--	--	--	--	--	--	--	--	--	--	--

NOTE: (a) + indicates PCR positive reaction in the pooled DNA of a given sample
(b) -- indicates PCR negative reaction in the pooled DNA of a given sample
Number of pollen trap progeny seeds per sample pool = 20