

# **Environmental Safety Studies**

- 1. Pollen flow study**
- 2. Cross pollination studies in Wild Species of Cotton**
- 3. Soil microflora**
- 4. Soil fauna**

## 1. Pollen Flow studies

Title	:	Pollen Flow studies
Objects	:	To find out travel distance of the pollen grains From last row of the transgenic crop to all the Four sides of plot <i>viz</i> North, South, East, and West. : To study the percentage of transgenic pollen Flows out and cross-pollinate with compatible Crops cultivated in and around the area.
Experiment conducted at	:	CICR, Panjari farm, Nagpur.
Date of study	:	During Kharif 2006
Date of submission of RCGM	:	The results were submitted to RCGM, DBT, New Delhi.

### Experimental Design:

The pollen flow trial was conducted as per the guidelines of RCGM, DBT, New Delhi. In this trial the transgenic seeds (BN-Bt) were sown in the centre for 50 Sq. mts. areas and surrounded by non-transgenic cotton variety in all four sides as refuge crop up to the distance of 30 meters.

The seeds of boll set in the non-transgenic male sterile lines crops were subjected to ELISA test to find cross-pollination and quantify the CRY protein expression. The bolls were collected randomly by row distance *viz.*, 1mt, 6mt, 11mt, 16mt, 21mt, 28mt and direction wise North, South, East and West. Fresh 10mg of samples were collected from bolls and ground well in the buffer to extract total protein. This total protein was subjected to test with lateral flow of ELISA to find out Cry 1 Ac protein presence. Total 72 samples were analyzed and results are presented below.

## **Results:**

- ✓ Total 72 numbers of samples were analyzed by ELISA test. The results were confirmed that the pollen flow up to the distance of 1 mt in North, West and East direction and up to 6 mt in South direction.
- ✓ More than 6 mt there was no contamination or cross-pollination by the transgenic pollen grains.
- ✓ These results clearly indicated that the maximum pollen flow in the field trial is up to 1-6 meters only.
- ✓ The frequency of out cross was approximately 1 % to Non-Bt cotton plants.

## **2. Cross pollination studies in Wild Species of Cotton**

Objective was to study the effect of cross pollination of BN Bt on wild species of Cotton

The study was conducted at wild species Garden, Central Institute for Cotton Research, Nagpur

### **Materials :**

Eleven wild species of *Gossypium* namely *G. anomalum*(B<sub>1</sub>) , *G. triphyllum*(B<sub>2</sub>), *G. capitivirides*(B<sub>3</sub>), *G. thurberi*(D<sub>1</sub>), *G. klotzschianum*(D<sub>3-k</sub>) , *G. davidsonii*(D<sub>3-d</sub>), *G. aridum*(D<sub>4</sub>), *G. raimondii*(D<sub>5</sub>), *G. lobatum*(D<sub>7</sub>) , *G. bickii*(G<sub>1</sub>) and *G. longicalyx*(F) as female parent each.

BNBt as male parent.

### **Experiment :**

Wild species were used as female parent while BN Bt was used as male parent in the cross pollination studies .On the day preceding pollination, the unopened buds of wild species were emasculated between 1400 – 1600 hrs in the afternoon and bagged. The next morning fresh pollen was collected from the field in which BN Bt is grown. This pollen was dusted on the stigma of emasculated flowers between 0800 – 1100 hrs and again bagged & labelled. Observations were recorded on percent fertilisation and seed set on the wild species upon pollination. In all, flowering of eleven (11) wild species synchronized with that of BN Bt and were utilized in the crossing program and a total of 863 pollinations were attempted. These are: *G. anomalum* , *G. triphyllum*, *G. capitivirides*, *G. thurberi*, *G. klotzschianum* , *G. davidsonii*, *G. aridum*, *G. raimondii*, *G. lobatum* , *G. bickii* and *G. longicalyx*.

### Hybridization between wild species of *Gossypium* and BNBt

SNo	Wild species (♀)	Pollinator (♂)	No. of flowers emasculated and pollinated	No of flowers retained after pollination	No of bolls formed	Hybrid seeds obtained
1.	<i>G. anomalum</i> (B <sub>1</sub> )	BNBt	110	0	0	0
2.	<i>G. triphyllum</i> (B <sub>2</sub> )	BNBt	77	0	0	0
3.	<i>G. capitata-virides</i> (B <sub>3</sub> )	BNBt	89	0	0	0
4.	<i>G. thurberi</i> (D <sub>1</sub> )	BNBt	97	0	0	0
5.	<i>G. klotzschianum</i> (D <sub>3-k</sub> )	BNBt	60	0	0	0
6.	<b><i>G. davidsonii</i></b> (D <sub>3-d</sub> )	BNBt	69	0	0	0
7.	<i>G. aridum</i> (D <sub>4</sub> )	BNBt	93	0	0	0
8.	<i>G. raimondii</i> (D <sub>5</sub> )	BNBt	95	0	0	0
9.	<i>G. lobatum</i> (D <sub>7</sub> )	BNBt	63	0	0	0
10.	<i>G. bickii</i> (G <sub>1</sub> )	BNBt	64	0	0	0
11.	<i>G. longicalyx</i> (F)	BNBt	46	0	0	0

### Results :

There was no fertilization and thereby no seed set on the wild species upon cross pollination with the BN Bt pollen.

Hence it could be concluded that there is no effect of cross-pollination of BN Bt on wild species of Cotton.

# **Transgenic Cotton expressing Bt CRY protein and its effects on soil microflora**

### **3. Soil microflora**

#### **Study Title:**

Transgenic Bikaneri Narma, BN-BT (*Gossypium hirsutum*) cotton expressing *cryIAc* gene: Effects on soil microflora.

#### **Objective:**

The objective of this study is to assess the safety of BN-BT cotton expressing *cryIAc* on soil microflora

#### **Summary:**

An important aspect of the biosafety assessment of genetically modified plant is to study its impact on soil ecosystem including changes in plant associated microflora. In the present investigation the effect of BN-BT plant expressing *Bacillus thuringiensis* (Bt) *cryIAc* on soil microflora was evaluated. BN-BT plant developed in CICR along with its non-BT counterpart was grown for obtaining various observations in the experimental farm of the institute. Soil samples were collected at set time points from the rhizosphere of the BT and non-BT plants and were analysed for the soil fungal and bacterial population. No significant differences in the rhizosphere microflora between BN-BT and its non-BT counterpart were observed.

#### **Materials and Methods:**

##### ***Soil sampling***

Soil samples were collected from the rhizosphere of BN-BT cotton and its non-BT counterpart grown at the CICR farm at 30, 90 and 150 days after sowing. Area of 1ft around the plant was marked and represented as the rhizosphere soil. Each rhizosphere soil sample was comprised of 5 core samples drawn from 5-6 inches deep and 3 inch wide bore made around the rhizosphere of 5 randomly selected BT plant. The soil was also drawn by the same procedure from the rhizosphere of non-BT plants too. The soil

from the two treatments was mixed thoroughly in separated containers. From this 0.5 kg soil was drawn as representative rhizosphere sample.

#### ***Determination of total bacterial and fungal population***

To determine the total bacterial and fungal populations, rhizosphere soil were homogenized in a pestle to break the clods. Samples were prepared in sterile distilled water by suspending 1 gm soil in 100 ml water and shaking the sample vigorously for 20 min on an orbital shaker at 250 rpm. The primary soil suspensions were serially diluted further and  $10^{-4}$  dilutions were plated on nutrient agar (NA) and Rose-Bengal supplemented potato dextrose agar (PDA) media in Petri plates to determine populations of bacterial and fungal micro flora respectively. The plates were incubated at 27-30°C for 3 and 7 days for bacteria and fungi respectively and observed for the appearance of colonies. The population count of the organisms was recorded after the stipulated incubation periods. The differences in the total bacterial and fungal populations in BN-BT and Non-BT rhizosphere were determined using SAS package with analysis of variance (ANOVA).

#### **Results:**

Total number of bacterial and fungal colonies was counted and analysis of variance was done to determine the effect of BN-BT cotton on soil microflora. The results of the bacterial study are presented in Table 1. Populations of rhizosphere bacteria went on increasing from 60 DAS to 150 DAS in both BN-BT and non-BT plants. Reason for this trend is not clear. However it might be possible that increased total root biomass with the passage of time, might be instrumental in supporting higher bacterial population (Table 1). No significant difference was however observed in the culturable bacterial population count between BN-BT and non-BT rhizosphere. Also no potential shift in the population levels of any type of bacterium was observed between the two treatments (Fig. 1). The types of colonies which grew from BN-BT cotton and non-BT cotton were similar. The bacterial strains viz., *Pseudomonas fluorescens*, *Bacillus* species, actinomycetes, etc were predominant in the rhizosphere of two plant types.

Table 1. Effect of BN-BT (*cryIAc*) cotton on soil rhizosphere bacterial population at three different crop stages at CICR farm

Treatment	Rhizosphere bacterial population (Cfu/gm soil)		
	Sampling time-point		
	29-08-06	29-10-06	29-01-07
BN-BT	1.96 x 10 <sup>5</sup>	1.59 x 10 <sup>6</sup>	3.67 x 10 <sup>6</sup>
BN-NBT	2.03 x 10 <sup>5</sup>	1.64 x 10 <sup>6</sup>	3.62 x 10 <sup>6</sup>
CD(p=0.05)			

The total fungal populations in the Rhizosphere of BN-BT and Non-BT cotton samples are presented in Table 2. Rhizosphere fungal population declined with the advancement of crop growth stage irrespective of the type of cotton grown. This could either be due to decline in the soil moisture content with the progress of the season or due to change in Temperature regime. However no significant difference was observed either in the population or type of fungi associated with the rhizosphere of BN-BT and its non-BT counterpart (Fig. 2 a-d). Species of *Aspergillus*, *Penicillium* and *Rhizopus* were the predominant fungi in rhizosphere of BN-BT and the non-BT cotton. Species of *Aspergillus* include *A. flavus*, *A. nidulans* and *A.* Besides these, species of *Fusarium*, *Trichoderma*, *Gliocladium*, *Myrothecium*, *Alternaria*, *Rhizoctonia*, *Paecilomyces*, non-sporulating strains were some of the other fungi common in the rhizosphere of both the types of plants.

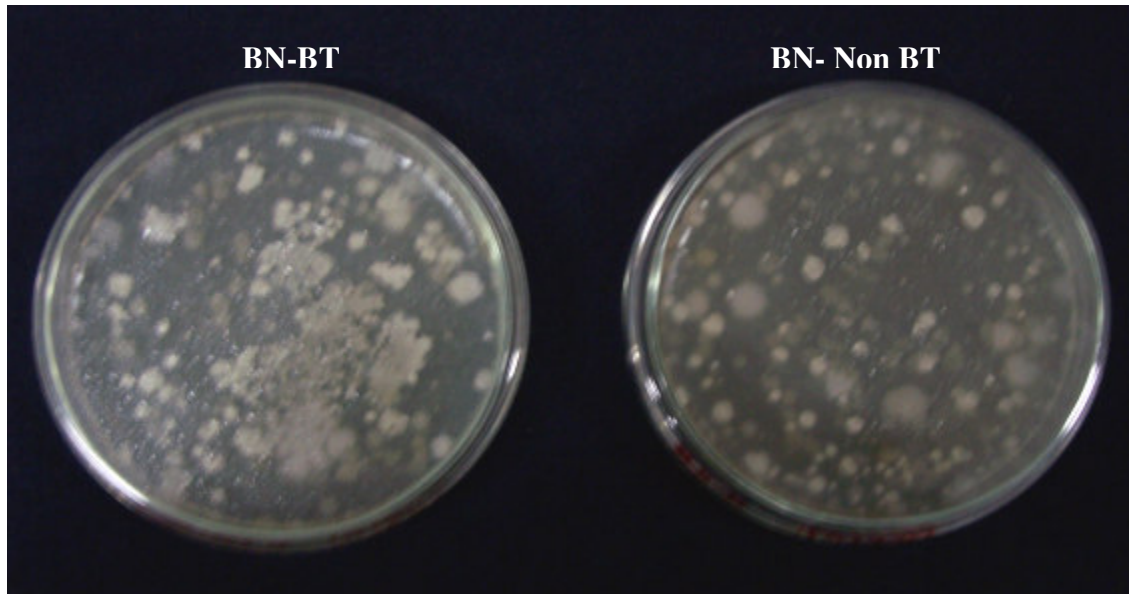
Table 2. Effect of BN-BT (*cryIAc*) cotton on soil rhizosphere fungal population at three different crop stages at CICR farm

Treatment	Rhizosphere fungal population ( x 10 <sup>5</sup> Cfu/gm soil)		
	Sampling time-point		
	29-08-06	29-10-06	29-01-07
BN-BT	4.06 x 10 <sup>5</sup>	1.63 x 10 <sup>5</sup>	1.13 x 10 <sup>5</sup>
BN-NBT	3.93 x 10 <sup>5</sup>	1.80 x 10 <sup>5</sup>	1.23 x 10 <sup>5</sup>
CD(p=0.05)			

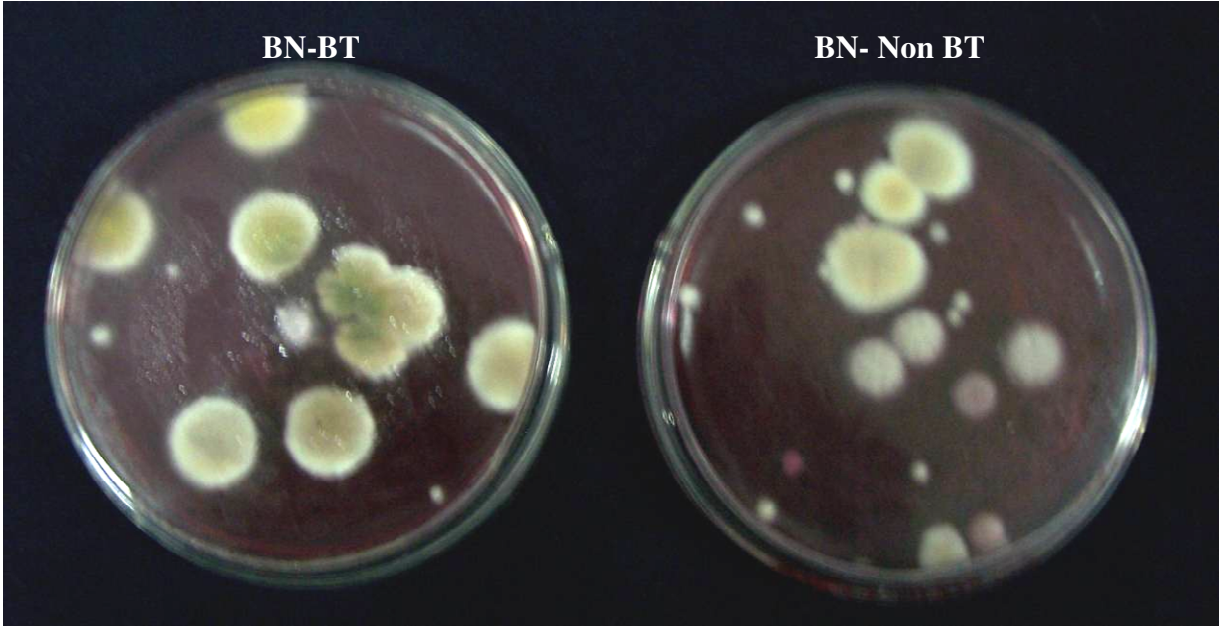
It is clearly apparent from the results of the microbial population analyses that BN-BT plants did not cause any change in the culturable soil bacteria and fungi. The results showed that the BN-BT cotton did not have any adverse effect on soil microflora and was safe to non-target organisms.

**Conclusion:**

Our findings demonstrated that the BN-BT transgenic cotton expressing *cryIAc* gene does not have any adverse effect on the soil microflora and was safe to non-target microbes, including fungi and bacteria.



**Fig. 1. Effect of BN-BT (*cryIAc*) cotton on soil rhizosphere bacterial population. No significant variability existed in Rhizosphere population of BN-BT vs NonBT cotton**



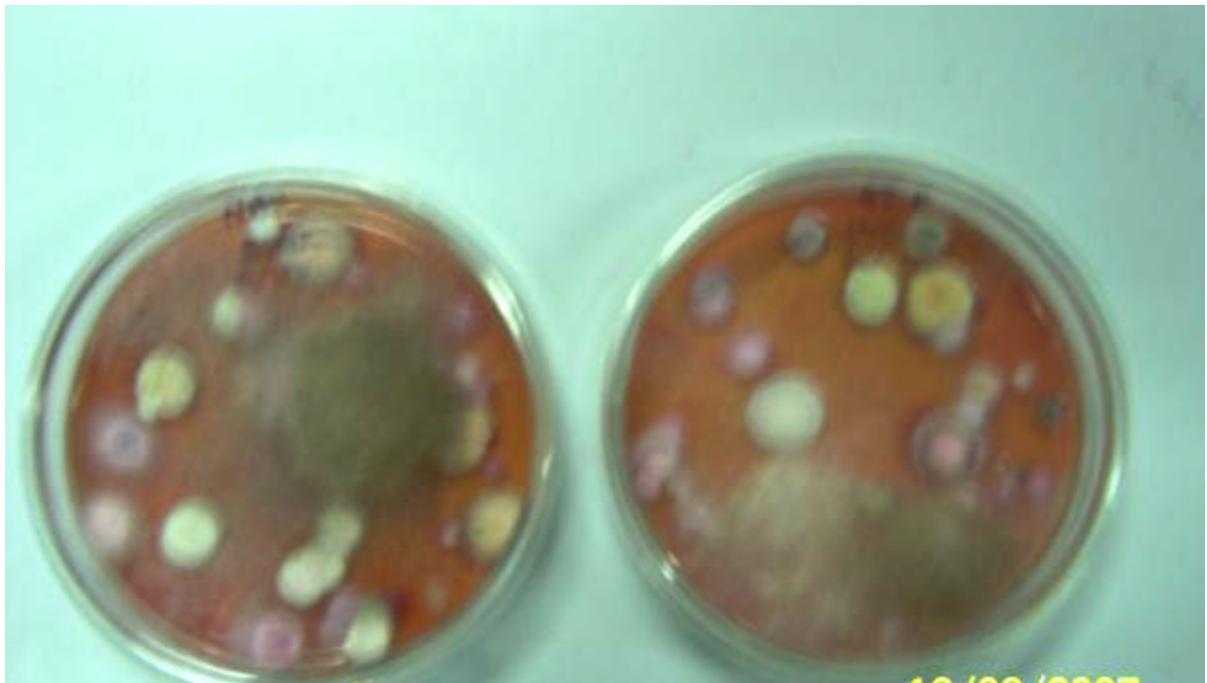
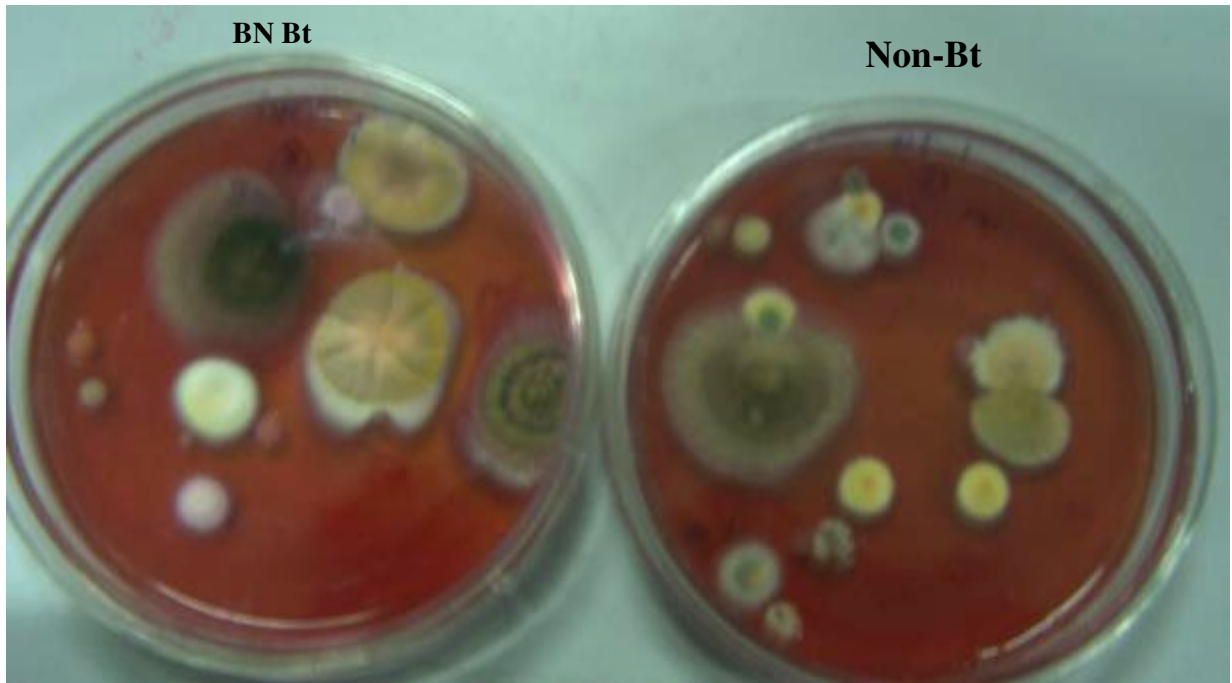


Fig. 2 a-d. Effect of BN-BT (*cryIAc*) cotton on soil rhizosphere fungal population. No significant variability existed in Rhizosphere mycoflora of BN-BT vs NonBT cotton in terms of colony population or fungal species.

**Transgenic Cotton expressing Bt CRY  
protein and its effects on soil fauna  
(earthworms)**

## **RCGM trial –Biosafety fauna (earthworms)**

### **Study Title:**

Transgenic Bt cotton plants expressing Cry protein and its effect on soil fauna especially earthworms.

### **Objectives:**

1. To study the effect of Bt and Non-Bt cultivars on soil fauna mainly earthworms
2. To study the effect of Bt and Non-Bt cultivars on dehydrogenase activity.

#### **I. Monitoring of earthworms (after cotton picking)**

Field extraction with 1% Formalin was adopted in 0.6 x 0.6 m area.

No activity of earthworms was noticed in Bt and Non- Bt plots.

#### **II. Dehydrogenase assay was done to determine the overall biological activity of the soil. The enzyme activity ranges from 17.2 to 28.4 mg / TPF / g soil /h.**

Treatment differences between cultivars were not significant. The results suggest no adverse effects of Bt cultivars on the soil biological activity.

However, the low dehydrogenase activity indicates the low biological activity mainly due to the low soil organic carbon and the calcareous nature of the soil and poor soil fertility status in rainfed condition.