Final Report on Project entitled:

"Studies on Soil Microflora of Bt and Non-Bt Cotton Fields: A Comparative Evaluation"

Submitted to:

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Introduction

Cotton is a very important fiber crop in India. Cotton provides a livelihood to more than 60 million people in India by way of support in agriculture, processing, and use of cotton in textiles. Cotton contributes 29.8% of the Indian agricultural gross domestic product, and nearly nine million hectares of land in India is used to produce 14.2 million bales of cotton lint (Barwale *et al.*, 2004). Indian cotton production is third in the world in quantity, although the productivity is substantially low. The major reason for the low productivity is damage caused by insect pest- *Helicoverpa armigera*, commonly referred to as American Bollworm. Other important lepidopteran insect pests of cotton in India are the pink bollworm (*Pectinophora gossypiella*), spotted bollworm (*Earias vittella*), spiny bollworm (*Earias insulana*) and tobacco caterpillar (*Spodoptera litura*). Nearly, Rs. 12 billion worth of pesticides are used in India to control just the bollworm complex of cotton.

Bt cotton was among the first GM crops to be commercialized in the mid-1990s. Mahyco (Maharashtra Hybrid Seed Company), in collaboration with Monsanto, has introduced Bt cotton technology in India. Bt cotton carries the Cry1Ac gene derived from the common soil bacterium *Bacillus thuringienesis* (Bt) var. *kurstaki*, which results in the expression of the Cry1 Ac protein that confers resistance to the bollworm complex. There is extensive information about microbial preparations of *Bacillus thuringiensis* sub sp. kurstaki (B.t.k.) containing Cry proteins, including the Cry1 Ac protein, that demonstrate that these proteins are non toxic to non-target organisms (US.EPA, 1988; Betz *et al.*, 2000). In another study, Palm *et al.*, (1994, 1996) indicated that Bt protein does not persist in the soil and is broken down in a matter of weeks. It can be clearly established that the Cry proteins are extremely selective for the lepidopteron insects, bind specifically to receptors on the midgut of lepidopteron insects and have no deleterious effect on beneficial/ non-target insects.

The introduction of transgenic crops has provided new approaches to improving crop quality and productivity but at the same time, these crops have aroused concerns about the safety of agricultural biotechnology in relation to human health and the environment. An important aspect of the biosafety assessment of genetically engineered plants is to study their impact on soil ecosystems including changes in the plant-associated microflora.

Therefore, in the present study we have assessed the effect of Bt cotton on soil microflora in terms of the total population of bacteria and fungi, in control (0 day), Bt and Non-Bt soil samples (50 and 150 days) by the dilution plating method. In addition to this, the Bt protein concentration in control, Bt and Non-Bt soil samples were estimated using Enzyme

Linked Immunosorbent Assays (ELISA). The soil samples that were analyzed were from six different states i.e. Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu of two consecutive crop seasons.

Objectives of the study

The Environmental safety of the transgenic Bt cotton crops being an issue of concern, it is important to study their impact on the soil ecosystem (microbial diversity), for which detailed comparative studies with the traditional cotton crops need to be undertaken. Keeping this in mind, the objectives of the present study are:

1. To determine the total soil microflora in soil samples taken from Bt and Non-Bt cotton fields.
2. Characterization of the dominant bacteria present in these soil samples.
3. To estimate Bt protein concentration in Bt- and Non-Bt cotton soils.

(i) Determination of the soil microbial diversity

In order to evaluate the impact of the transgenic or Bt cotton crops on soil ecosystems, extensive studies were carried out to determine the soil microbial diversity. In all the studies carried out in present work, we have used the soil samples from six different states i.e. Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu of two consecutive crop seasons. The soil samples provided by the JK Agri Genetics Ltd. of the above mentioned states were described as control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of I crop season and control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of II crop season respectively. Various parameters such as pH, electrical conductivity, organic matter content, etc. of the above mentioned soil samples of both the crop seasons have been provided by JK Agri Genetics Ltd. (Table 1 & Table 2).

(a) Total colony counts:

For the determination of the total number of bacterial and fungal populations present in the soil samples (control, Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere), suspensions of the soil samples were prepared in sterile normal saline by suspending 1g of soil sample in 10ml of saline. The samples were then vortexed vigorously and placed on a shaker for 30 minutes at 215 rpm. These primary soil suspensions were serially diluted further and appropriate dilutions were plated on various media such as soyabean casein digest agar (tryptone soya agar or TSA) and potato dextrose agar (PDA). The plates were incubated at 30^{0}C for 2-3 days. The total numbers of colonies were counted after the completion of the incubation period and the numbers of colony forming units (CFUs) were determined per gram of soil. The data for the total number of CFUs obtained on various media for the control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of I crop season and control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of II crop season, soil samples of Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu are presented in tables 3-8 and 9-14, respectively. As it is clear from tables 3-14, all the colony counts were obtained in the range of 10^{6} CFU/g of soil both in Bt as well as Non-Bt soil samples for both crop seasons. These results indicate that no particular difference was observed in any of the samples examined on the various media used.

(b) Determination of the predominant types of colonies:

In order to determine the total microbial diversity present in the Bt and Non-Bt cotton soil environments; the number of different types of colonies present on the plates were determined purely based on colony morphology. Also, the percentage of the predominant types of colonies was calculated from the total colony counts on the plates. Each morphologically different type of colony was characterized in terms of colony characteristics such as color, shape, texture, etc of the colonies. Therefore, about 700-800 colonies isolated on various media were characterized in this manner.

The data for the predominant types of colonies obtained on Tryptone soya agar (TSA) of all the soil samples i.e. control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 days) and soil samples Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (150 days) of I crop season are presented in Table 15-20 and Table 21-26, respectively. Similarly, soil samples of control (0 day), Bt rhizosphere, Non-Bt rhizosphere (50 days) and Bt rhizosphere, Non-Bt rhizosphere (150 days) of II crop season of Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu are presented in Table 27-32 and Table 33-38, respectively.

(c) Preservation of the microorganisms:

Each colony was preserved in 10% glycerol stocks at -70^0 C. Each colony was given a unique and specific preservation number from which it can be later revived and used for identification purposes.

(d) Identification of the microorganisms:

The identification of the cultures was on the basis of a number of biochemical tests according to Bergey's manual of Systematic Bacteriology (Vol. 1 & 2, 1986). Table 39 & 40 shows the genus level identification tests of the most dominant bacteria isolated from control, Bt and Non-Bt soil samples of the I crop season and II crop season respectively. The most prominent members of bacteria obtained in control, Bt and Non-Bt soil samples belonged to members of *Bacillus* sp. Some of the other species of bacteria isolated from Bt and Non-Bt soil samples included those of *Arthrobacter* sp., *Kurthia* sp., *Micrococcus* sp. and *Alcaligenes* sp.

(ii) Estimation of Bt protein (Cry1Ac) concentration in Bt and Non Bt soils

E. Ramachandran (Scientist, JK Agri Genetics Ltd.), using Envirologix Quantiplate TM Kit for Cry1Ab/Cry1Ac at Institute of Microbial Technology (IMTECH) Chandigarh has done estimation of Bt protein (Cry1Ac) concentration in Bt and Non-Bt soils for I crop season. The estimation of Bt protein (Cry1 Ac) concentration in soil samples of II crop season was done using Cry1 Ac/ Ab Elisa Kit (Amar Immunodiagnostics), provided by JK Agri Genetics Ltd. The level of Cry1Ac protein in all soil samples was evaluated using Enzyme linked immunosorbent assays (ELISA).

Procedure:

Put 0.5 g of soil sample in 1.5 ml eppendorf tube each

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Add 1 ml extraction buffer in each tube

↓

Shake well

↓

Vortex each tube for 2 min. approx.

↓

Centrifuge it at 12,000 rpm for 2-3 min.

↓

Load 100 µl of each sample in Cry1Ab/Ac coated plate

↓

Incubate for 15 min. at room temperature

↓

Add Cry1 Ab/Ac enzyme conjugate (100 µl) in each well

↓

Incubate for 1h at room temperature

↓

Washing with Phosphate Buffer Saline (5-6 times)

↓

After drying, add 100 µl of substrate in each well

↓

Incubate for 30 min. at room temp.

↓

Add Stop Solution

↓

Take reading at 450 nm in ELISA reader

Table 41 represents the readings of blank, 1.5ng, 10ng and 25ng (calibrators available in kit for Cry1Ac proteins in triplicate) and soil samples i.e. control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu, of I crop season.

Table 42 represents the readings of blank, negative and positive control (available in kit for Cry1Ac proteins in duplicate) and soil samples i.e., control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu, of II crop season.

It was observed that there was no detectable amount of Cry1 Ac protein present in the soil samples of both the crop seasons.

Conclusions

On the basis of present study, it can be concluded that the soil microbial diversity data on total colony counts of culturable microorganisms revealed no particular difference in control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of I crop season and control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of II crop season of any of the state i.e. Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu. About 700-800 bacterial cultures from control, Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere soil samples of both the crop seasons were isolated on tryptone soya agar (TSA) and preserved as glycerol stocks at $-70^{\circ}C$. The unique colony characteristics for all the isolated colonies have been recorded and the most predominant cultures from TSA plates have been identified. The predominant types of colonies obtained on TSA plates of control, Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere soil samples of I and II crop season belonged to the genera of *Bacillus* sp. The Bt protein (Cry1 Ac) concentration in Bt and Non-Bt soil samples estimated and it has been observed that there was no detectable amount of Cry1Ac protein in the soil samples of both the crop seasons.

References

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Table 1: Various parameters of soil samples (of I crop season) from six different states i.e. Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu (Provided by JK Agri Genetics Ltd.)

Parameters	Units	Rajasthan	Gujarat	Maharashtra	Karnataka	Andhra Pradesh	Tamil Nadu
pH	(---)	9.04	7.73	9.00	7.50	8.36	8.91
Electrical Conductivity	(milli.mho)	0.094	0.041	0.084	0.046	0.186	0.094
Organic Matter	(%)	0.98	1.01	1.76	0.92	1.63	1.59
Nitrogen	(%)	0.026	0.023	0.029	0.033	0.056	0.041
Phosphorus	(ppm)	12	7	13	8	11	9
Potassium	(ppm)	139	161	182	101	157	108
Chlorides	(%)	0.0709	0.0561	0.079	0.067	0.106	0.106
Sodium Absorption Ratio	(---)	1.59	2.04	1.28	3.10	4.00	1.64

Table 2: Various parameters of soil samples (of II crop season) from six different states i.e. Rajasthan, Gujarat, Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu (Provided by JK Agri Genetics Ltd.)

Parameters	Units	Rajasthan	Gujarat	Maharashtra	Karnataka	Andhra Pradesh	Tamil Nadu
pH (20% solution)	(---)	8.75	8.71	8.64	8.66	8.57	8.76
Electrical Conductivity	(milli.mho)	0.440	0.166	0.185	0.262	0.205	0.166
Organic Matter	(%)	0.70	0.81	0.81	1.32	0.862	0.899
Nitrogen	(%)	290	340	440	490	480	378
Phosphorus	(ppm)	18.70	12.50	13.10	13.50	15.80	12.42
Potassium	(ppm)	313	91	42	56	159	43
Chlorides	(%)	160	71	86	78	75	96
Total Dissolved solids	(ppm)	260	102	111	157	135	109

Table 3: Total number of bacterial colonies on different media from control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of Rajasthan soil samples of I crop season

Media	Colony Forming Units/g soil								
	O day	50 days				150 days			
	Control	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$0.89x10^6$	$1.0x10^6$	$0.54x10^6$	$1.9x10^6$	$3.1x10^6$	$3.7x10^6$	$5.6x10^6$	$1.0x10^6$	$3.2x10^6$
Potato Dextrose Agar (PDA)	$0.38x10^6$	$0.44x0^6$	$0.37x10^6$	$0.33x10^6$	$1.0x10^6$	$1.8x10^6$	$3.2x10^6$	$0.59x10^6$	$1.0x10^6$

Table 4: Total number of bacterial colonies on different media from control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of Gujarat soil samples of I crop season

Media	Colony Forming Units/g soil								
	O day	50 days				150 days			
	Control	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$1.0x10^6$	$1.0x10^6$	$1.6x10^6$	$1.9x10^6$	$1.6x10^6$	$4.4x10^6$	$1.4x10^6$	$5.0x10^6$	$3.3x10^6$
Potato Dextrose Agar (PDA)	$0.32x10^6$	$0.49x0^6$	$0.35x10^6$	$0.38x10^6$	$0.42x10^6$	$0.93x10^6$	$0.76x10^6$	$0.93x10^6$	$0.94x10^6$

Table 5: Total number of bacterial colonies on different media from control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of Maharashtra soil samples of I crop season

Media	Colony Forming Units/g soil								
	O day	50 days				150 days			
	Control	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere
Tryptone soya agar (TSA)	$1.2x10^6$	$1.2x10^6$	$2.0x10^6$	$1.0x10^6$	$2.6x10^6$	$6.4x10^6$	$4.9x10^6$	$6.8x10^6$	$2.2x10^6$
Potato Dextrose Agar (PDA)	$0.31x10^6$	$0.42x0^6$	$0.31x10^6$	$0.31x10^6$	$0.21x10^6$	$1.2x10^6$	$1.0x10^6$	$1.9x10^6$	$1.1x10^6$

Table 6: Total number of bacterial colonies on different media from control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of Karnataka soil samples of I crop season

Media	Colony Forming Units/g soil								
	O day	50 days				150 days			
	Control	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$1.6x10^6$	$2.6x10^6$	$0.95x10^6$	$1.2x10^6$	$0.93x10^6$	$3.4x10^6$	$4.6x10^6$	$2.4x10^6$	$2.6x10^6$
Potato Dextrose Agar (PDA)	$0.51x0^6$	$0.73x10^6$	$0.78x10^6$	$0.65x10^6$	$0.72x10^6$	$0.52x10^6$	$0.54x10^6$	$0.87x10^6$	$0.56x10^6$

Table 7: Total number of bacterial colonies on different media from control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of Andhra Pradesh soil samples of I crop season

Media	Colony Forming Units/g soil								
	O day	50days				150 days			
	Control	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere
Tryptone soya Agar (TSA)	$1.4x10^6$	$1.4x10^6$	$1.7x10^6$	$1.3x10^6$	$2.2x10^6$	$3.3x10^6$	$1.1x10^6$	$3.3x10^6$	$1.2x10^6$
Potato Dextrose Agar (PDA)	$0.56x10^6$	$0.60x10^6$	$0.44x10^6$	$0.49x10^6$	$0.53x10^6$	$0.59x10^6$	$0.19x10^6$	$0.20x10^6$	$0.13x10^6$

Table 8: Total number of bacterial colonies on different media from control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) of Tamil Nadu soil samples of I crop season

Media	Colony Forming Units/g soil								
	O day	50 days				150 days			
	Control	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere	Bt	Bt rhizosphere	Non-Bt	Non-Bt rhizosphere
Tryptone soya agar (TSA)	$2.0x10^6$	$2.0x10^6$	$1.8x10^6$	$3.5x10^6$	$3.3x10^6$	$2.1x10^6$	$1.1x10^6$	$1.2x10^6$	$1.8x10^6$
Potato Dextrose Agar (PDA)	$0.45x10^6$	$1.5x0^6$	$0.48x10^6$	$0.27x10^6$	$0.36x10^6$	$0.15x10^6$	$0.34x10^6$	$0.31x10^6$	$0.15x10^6$

Table 9: Total number of bacterial colonies on different media from control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of Rajasthan soil samples of II crop season

Media	Colony Forming Units/g soil				
	O day	50 days		150 days	
	Control	Bt rhizosphere	Non-Bt rhizosphere	Bt rhizosphere	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$1.0x10^6$	$1.3x10^6$	$1.5x10^6$	$3.9 x10^6$	$3.1 x10^6$
Potato Dextrose Agar (PDA)	$0.35x10^6$	$0.34x10^6$	$0.31x10^6$	$1.9 x10^6$	$1.0 x10^6$

Table 10: Total number of bacterial colonies on different media from control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of Gujarat soil samples of II crop season

Media	Colony Forming Units/g soil				
	O day	50 days		150 days	
	Control	Bt rhizosphere	Non-Bt rhizosphere	Bt rhizosphere	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$0.8x10^6$	$1.0x10^6$	$1.5x10^6$	$1.3 x10^6$	$2.0 x10^6$
Potato Dextrose Agar (PDA)	$0.3x10^6$	$0.44x10^6$	$0.52x10^6$	$0.63 x10^6$	$0.52 x10^6$

Table 11: Total number of bacterial colonies on different media from control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of Maharashtra soil samples of II crop season

Media	Colony Forming Units/g soil				
	O day	50 days		150 days	
	Control	Bt rhizosphere	Non-Bt rhizosphere	Bt rhizosphere	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$0.81x10^6$	$1.7x10^6$	$1.3x10^6$	$3.1 x10^6$	$3.3 x10^6$
Potato Dextrose Agar (PDA)	$0.3x10^6$	$0.32x10^6$	$0.45x10^6$	$1.0 x10^6$	$1.1 x10^6$

Table 12: Total number of bacterial colonies on different media from control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of Karnataka soil samples of II crop season

Media	Colony Forming Units/g soil				
	O day	50 days		150 days	
	Control	Bt rhizosphere	Non-Bt rhizosphere	Bt rhizosphere	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$1.8x10^6$	$2.8x10^6$	$2.7x10^6$	$3.1 x10^6$	$2.4 x10^6$
Potato Dextrose Agar (PDA)	$0.6x10^6$	$0.74x10^6$	$0.82x10^6$	$0.59 x10^6$	$0.68 x10^6$

Table 13: Total number of bacterial colonies on different media from control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of Andhra Pradesh soil samples of II crop season

Media	Colony Forming Units/g soil				
	O day	50 days		150 days	
	Control	Bt rhizosphere	Non-Bt rhizosphere	Bt rhizosphere	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$1.2x10^6$	$1.6x10^6$	$1.6x10^6$	$1.3 x10^6$	$1.4 x10^6$
Potato Dextrose Agar (PDA)	$0.61x10^6$	$0.86x10^6$	$0.72x10^6$	$0.82 x10^6$	$0.65 x10^6$

Table 14: Total number of bacterial colonies on different media from control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) of Tamil Nadu soil samples of II crop season

Media	Colony Forming Units/g soil				
	O day	50 days		150 days	
	Control	Bt rhizosphere	Non-Bt rhizosphere	Bt rhizosphere	Non-Bt rhizosphere
Tryptone Soya Agar (TSA)	$1.8x10^6$	$2.5x10^6$	$2.1x10^6$	$2.0 x10^6$	$1.9 x10^6$
Potato Dextrose Agar (PDA)	$0.49x10^6$	$0.62x10^6$	$0.76x10^6$	$0.53 x10^6$	$0.55 x10^6$

Table 15: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 days) of Rajasthan soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	4 types	Colony 1~ 85% Colony 2~ 10%
#2	Bt	6 types	Colony 1~ 75% Colony 2~ 15%
#3	Bt rhizosphere	3 types	Colony 1~ 35% Colony 2~ 35%
#4	Non-Bt	6 types	Colony 1~ 40% Colony 2~ 30%
#5	Non-Bt rhizosphere	2 types	Colony 1~ 65% Colony 2~ 30%

Table 16: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 days) of Gujarat soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	5 types	Colony 1~ 35% Colony 2~ 25%
#2	Bt	6 types	Colony 1~ 65% Colony 2~15%
#3	Bt rhizosphere	6 types	Colony 1~ 55% Colony 2~ 25%
#4	Non-Bt	4 types	Colony 1~ 65% Colony 2~ 30%
#5	Non-Bt rhizosphere	4 types	Colony 1~ 90% Colony 2~ 5%

Table 17: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 days) of Maharashtra soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	6 types	Colony 1~ 65% Colony 2~10%
#2	Bt	7 types	Colony 1~ 80% Colony 2~ 10%
#3	Bt rhizosphere	7 types	Colony 1~ 75% Colony 2~ 5%
#4	Non-Bt	7 types	Colony 1~ 55% Colony 2~ 20%
#5	Non-Bt rhizosphere	5 types	Colony 1~ 95%

Table 18: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 days) of Karnataka soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	6 types	Colony 1~ 60% Colony 2~ 20%
#2	Bt	6 types	Colony 1~ 60% Colony 2~ 30%
#3	Bt rhizosphere	4 types	Colony 1~ 85% Colony 2~ 5%
#4	Non-Bt	3 types	Colony 1~ 65% Colony 2~ 30%
#5	Non-Bt rhizosphere	5 types	Colony 1~ 55% Colony 2~ 15%

Table 19: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 days) of Andhra Pradesh soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	13 types	Colony 1~ 45% Colony 2~ 25%
#2	Bt	10 types	Colony 1~ 60% Colony 2~ 20%
#3	Bt rhizosphere	10 types	Colony 1~ 40% Colony 2~ 40%
#4	Non-Bt	7 types	Colony 1~ 60% Colony 2~ 20%
#5	Non-Bt rhizosphere	5 types	Colony 1~ 60% Colony 2~ 35%

Table 20: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt hizosphere (50 days) of Tamil Nadu soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	8 types	Colony 1~ 60% Colony 2~ 35%
#2	Bt	5 types	Colony 1~ 75% Colony 2~ 10%
#3	Bt rhizosphere	7 types	Colony 1~ 70% Colony 2~ 10%
#4	Non-Bt	7 types	Colony 1~ 55% Colony 2~ 40%
#5	Non-Bt rhizosphere	6 types	Colony 1~ 75% Colony 2~ 20%

Table 21: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (150 days) of Rajasthan soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt	7 types	Colony 1~ 70% Colony 2~ 20%
#3	Bt rhizosphere	9 types	Colony 1~ 35% Colony 2~ 30%
#4	Non-Bt	4 types	Colony 1~ 45% Colony 2~ 30%
#5	Non-Bt rhizosphere	7 types	Colony 1~ 40% Colony 2~ 40%

Table 22: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (150 days) of Gujarat soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt	8 types	Colony 1~ 50% Colony 2~ 15%
#3	Bt rhizosphere	5 types	Colony 1~ 45% Colony 2~ 30%
#4	Non-Bt	3 types	Colony 1~ 45% Colony 2~ 45%
#5	Non-Bt rhizosphere	6 types	Colony 1~ 40% Colony 2~ 30%

Table 23: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (150 days) of Maharashtra soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt	5 types	Colony 1~ 55% Colony 2~ 35%
#3	Bt rhizosphere	4 types	Colony 1~ 70% Colony 2~ 15%
#4	Non-Bt	4 types	Colony 1~ 40% Colony 2~ 25%
#5	Non-Bt rhizosphere	5 types	Colony 1~ 80% Colony 2~ 15%

Table 24: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (150 days) of Karnataka soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt	7 types	Colony 1~ 45% Colony 2~ 25%
#3	Bt rhizosphere	5 types	Colony 1~ 85% Colony 2~ 15%
#4	Non-Bt	9 types	Colony 1~ 70% Colony 2~ 5%
#5	Non-Bt rhizosphere	5 types	Colony 1~ 85% Colony 2~ 10%

Table 25: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (150 days) of Andhra Pradesh soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt	10 types	Colony 1~ 70% Colony 2~ 5%
#3	Bt rhizosphere	7 types	Colony 1~ 70% Colony 2~20%
#4	Non-Bt	6 types	Colony 1~ 90% Colony 2~ 5%
#5	Non-Bt rhizosphere	8 types	Colony 1~ 50% Colony 2~ 45%

Table 26: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (150 days) of Tamil Nadu soil samples of I crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt	7 types	Colony 1~ 35% Colony 2~ 25%
#3	Bt rhizosphere	5 types	Colony 1~ 65% Colony 2~ 20%
#4	Non-Bt	7 types	Colony 1~ 60% Colony 2~ 25%
#5	Non-Bt rhizosphere	4 types	Colony 1~ 50% Colony 2~ 20%

Table 27: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 days) of Rajasthan soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	4 types	Colony 1~ 60% Colony 2~ 25%
#2	Bt rhizosphere	6 types	Colony 1~ 50% Colony 2~ 15%
#3	Non-Bt rhizosphere	5 types	Colony 1~ 95% Colony 2~ 2%

Table 28: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 days) of Gujarat soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	5 types	Colony 1~ 75% Colony 2~ 11%
#2	Bt rhizosphere	4 types	Colony 1~ 45% Colony 2~ 45%
#3	Non-Bt rhizosphere	4 types	Colony 1~ 50% Colony 2~ 30%

Table 29: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 days) of Maharashtra soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	4 types	Colony 1~ 40% Colony 2~ 20%
#2	Bt rhizosphere	5 types	Colony 1~ 35% Colony 2~ 20%
#3	Non-Bt rhizosphere	8 types	Colony 1~ 25% Colony 2~ 20%

Table 30: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 days) of Karnataka soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	5 types	Colony 1~ 50% Colony 2~ 40%
#2	Bt rhizosphere	5 types	Colony 1~ 50% Colony 2~ 25%
#3	Non-Bt rhizosphere	6 types	Colony 1~ 60% Colony 2~ 25%

Table 31: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 days) of Andhra Pradesh soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	4 types	Colony 1~ 45% Colony 2~ 40%
#2	Bt rhizosphere	4 types	Colony 1~ 55% Colony 2~ 35%
#3	Non-Bt rhizosphere	5 types	Colony 1~ 70% Colony 2~ 15%

Table 32: The percentage of the tentative predominant types of colonies obtained on TSA plates in Control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 days) of Tamil Nadu soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#1	Control	4 types	Colony 1~ 65% Colony 2~ 15%
#2	Bt rhizosphere	5 types	Colony 1~ 75% Colony 2~ 15%
#3	Non-Bt rhizosphere	4 types	Colony 1~ 75% Colony 2~ 9%

Table 33: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt rhizosphere and Non-Bt rhizosphere (150 days) of Rajasthan soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt rhizosphere	8 types	Colony 1~ 60% Colony 2~ 25%
#3	Non-Bt rhizosphere	5 types	Colony 1~ 80% Colony 2~ 10%

Table 34: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt rhizosphere and Non-Bt rhizosphere (150 days) of Gujarat soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt rhizosphere	4 types	Colony 1~ 65% Colony 2~ 15%
#3	Non-Bt rhizosphere	5 types	Colony 1~ 65% Colony 2~ 25%

Table 35: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt rhizosphere and Non-Bt rhizosphere (150 days) of Maharashtra soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt rhizosphere	9 types	Colony 1~ 40% Colony 2~ 30%
#3	Non-Bt rhizosphere	7 types	Colony 1~ 75% Colony 2~ 15%

Table 36: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt rhizosphere and Non-Bt rhizosphere (150 days) of Karnataka soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt rhizosphere	7 types	Colony 1~ 55% Colony 2~ 30%
#3	Non-Bt rhizosphere	7 types	Colony 1~ 60% Colony 2~ 30%

Table 37: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt rhizosphere and Non-Bt rhizosphere (150 days) of Andhra Pradesh soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt rhizosphere	4 types	Colony 1~ 75% Colony 2~ 20%
#3	Non-Bt rhizosphere	4 types	Colony 1~ 70% Colony 2~ 20%

Table 38: The percentage of the tentative predominant types of colonies obtained on TSA plates in Bt rhizosphere and Non-Bt rhizosphere (150 days) of Tamil Nadu soil samples of II crop season

Soil sample No.	Soil sample type	Types of colonies	Percentage of predominant types of colonies
#2	Bt rhizosphere	6 types	Colony 1~ 60% Colony 2~ 30%
#3	Non-Bt rhizosphere	5 types	Colony 1~ 55% Colony 2~ 30%

Table 39: Genus level identification tests of the dominant bacteria isolated from control, Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere soil samples of I crop season

S. No.	Culture Designation	Gram Staining (+/-, size, shape)	Spore (+/-, position, shape, bulging)	Catalase	Oxidase	Anaerobic growth	O/F	Acid from glucose	Motility	Identified as	Type of soil
1	AI 12	+, long rod	+, subterminal, oval and round,-	+	+	+	-	W	+	*Bacillus* sp	C
2	AI 48	+, moderate rod	+, subterminal, oval ,-	+	+	-	-	+	+	*Bacillus* sp	Btr
3	AI 62	+, moderate rod	+, subterminal, oval ,+	+	+	+	-	+	+	*Bacillus* sp	Btr
4	AI 66	+, moderate rod	-	+	-	-	-	-	+	*Kurthia* sp	Btr, N-Btr
5	AI 103	+, moderate rod	+,terminal,-	+	+	-	-	+	+	*Bacillus* sp	N-Btr
6	RI 1	+, long rod	+,subterminal, -	+	+	-	-	W	+	*Bacillus* sp	C
7	RI 28	+, moderate rod	+,subterminal, -	+	+	-	F	W	+	*Bacillus* sp	Btr, N-Btr
8	GI 7	_, very short rod	-	+	+	-	-	+	+	*Alcaligenes* sp.	C, Bt, Btr

S. No.	Culture Designation	Gram Staining (+/-, size, shape)	Spore (+/-, position, shape, bulging)	Catalase	Oxidase	Anaerobic growth	O/F	Acid from glucose	Motility	Identified as	Type of soil
9	GI 14	+, cocci in groups and pairs	-	+	-	-	-	+	-	*Micrococcus sp*	C, N-Btr
10	GI 23	+, moderate rod	+,subterminal, -	+	+	+	-	+	+	*Bacillus* ***sp***	Btr, N-Bt
11	GI 26	+, short rod	-	+	-	-	-	-	+	*Arthrobacter* sp.	Btr, N-Bt, N-Btr
12	KI 4	+, moderate rod	+, terminal, +	+	+	+	-	+	+	*Bacillus* sp.	C, Bt, Btr, N-Bt, N-Btr
13	KI 5	+, long rod	+, central, oval,-	+	+	+	F	+	+	*Bacillus* sp.	C
14	KI 8	+, short rod	-	+	-	-	-	W	+	*Arthrobacter* sp.	C, N-Btr
15	MI 1	+, short rod	-	+	-	-	-	W	+	*Arthrobacter* sp.	C, Btr, N-Bt, N-Btr
16	MI 5	+, moderate rod	+, subterminal, oval, +	+	+	+	-	+	+	*Bacillus* sp.	C
17	MI 20	+, long rod	+, subterminal, +	+	+	+	-	+	+	*Bacillus* sp.	Bt

S. No.	Culture Designation	Gram Staining (+/-, size, shape)	Spore (+/-, position, shape, bulging)	Catalase	Oxidase	Anaerobic growth	O/F	Acid from glucose	Motility	Identified as	Type of soil
18	MI 32	+, moderate rod	+, subterminal, +	+	-	+	-	+	+	*Bacillus* sp.	Btr
19	MI 61	+, moderate rod	+, subterminal, +	+	+	-	-	+	+	*Bacillus* sp.	N-Btr
20	TI 1	+, long rod	+, central and subterminal, +	+	+	+	-	+	+	*Bacillus* sp.	C, Bt, N-Bt, N-Btr
21	TI 2	+, moderate rod	+, central, round and oval, +	+	-	-	-	W	+	*Bacillus* sp.	C
22	TI 6	+, moderate rod	+, subterminal, +	+	+	-	-	W	+	*Bacillus* sp.	C
23	TI 9	+, moderate rod	+, central, oval, +	+	+	+	F	+	+	*Bacillus* sp.	C, Bt, Btr, N-Bt, N-Btr
24	TI 32	+, moderate rod	+, terminal, +	+	-	+	F	+	+	*Bacillus* sp.	Bt r, N-Btr

O=Oxidative, F=Fermentative
W=Weakly Positive
C=Control
Btr=Bt rhizosphere
N-Bt=Non-Bt
N-Btr=Non-Bt rhizosphere

Table 40: Genus level identification tests of the dominant bacteria isolated from control, Bt rhizosphere and Non-Bt rhizosphere soil samples of II crop season

S. No.	Culture Designation	Gram Staining (+/-, size, shape)	Spore (+/-, position, shape, bulging)	Catalase	Oxidase	Anaerobic growth	O/F	Acid from glucose	Motility	Identified as	Type of soil
1	RII 1	+, short rod	-	+	-	-	-	-	+	*Kurthia* sp	C, Btr, N-Btr
2	APII 1	+, long rod	+, central, oval, +	+	-	-	F	+	+	*Bacillus* sp	C, Btr, N-Btr
3	APII 3	+, long rod	+, subterminal, oval, +	+	+	-	-	+	+	*Bacillus* sp	C, Btr
4	APII 5	+, very short rod	+, central, oval, +	+	+	-	F	+	+	*Bacillus* sp.	C, Btr, N-Btr
5	GII 1	-, very short rod	-	+	+	-	-	+	+	*Alcaligenes* sp.	C, Btr, N-Btr
6	GII 10	-, moderate rod	-	+	+	-	-	-	+	*Alcaligenes* sp.	C, Btr, N-Btr
7	MII 5	+, moderate rod	+, central, oval, +	+	+	-	-	+	+	*Bacillus* sp.	C, Btr
8	MII 9	+, very short rod	+, subterminal, oval, +	+	+	-	-	+	+	*Bacillus* sp.	Btr, N-Btr
9	MII 13	+, moderate rod	+, central, oval, +	+	+	-	-	+	+	*Bacillus* sp.	Btr, N-Btr

S. No.	Culture Designation	Gram Staining (+/-, size, shape)	Spore (+/-, position, shape, bulging)	Catalase	Oxidase	Anaerobic growth	O/F	Acid from glucose	Motility	Identified as	Type of soil
10	KII 1	+, short rod	+, central, oval, +	+	+	-	-	+	+	*Bacillus* sp.	C, Btr, N-Btr
11	KII 9	+, cocci in groups	-	+	-	-	-	+	-	*Micrococcus* sp.	C, Btr, N-Btr
12	TII 1	_, very short rod	-	+	+	-	-	-	+	*Alcaligenes* sp.	C, Btr, N-Btr
13	TII 2	+, long rod	+, subterminal, oval, +	+	+	-	F	+	+	*Bacillus* sp.	C, Btr, N-Btr
14	K_{150} 1	+, long rod	+, central, oval, +	+	+	-	-	+	+	*Bacillus* sp.	Btr, N-Btr
15	K_{150} 2	+, moderate rod	-	+	-	-	-	-	+	*Kurthia* sp	Btr, N-Btr
16	T_{150} 2	+, long rod	+, central, oval, +	+	+	-	F	+	+	*Bacillus* sp.	Btr, N-Btr
17	T_{150} 7	+, cocci	-	+	-	-	-	+	-	*Micrococcus* sp.	Btr, N-Btr
18	M_{150} 1	+, long rod	+, subterminal, oval, +	+	+	-	-	+	+	*Bacillus* sp.	Btr, N-Btr
19	M_{150} 2	+, very short rod	-	+	-	-	-	-	+	*Arthobacter* sp.	Btr, N-Btr
20	R_{150} 1	+, long rod	+, central, oval, +	+	+	-	-	+	+	*Bacillus* sp.	Btr, N-Btr
21	R_{150} 11	+, cocci, tetrad form	-	+	-	-	-	-	-	*Micrococcus* sp.	Btr, N-Btr

S. No.	Culture Designation	Gram Staining (+/-, size, shape)	Spore (+/-, position, shape, bulging)	Catalase	Oxidase	Anaerobic growth	O/F	Acid from glucose	Motility	Identified as	Type of soil
22	AP_{150} 1	+, long rod	+, subterminal, oval, +	+	+	-	F	+	+	*Bacillus* sp.	Btr, N-Btr
23	AP_{150} 2	+, cocci in groups	-	+	-	-	-	+	-	*Micrococcus* sp.	Btr, N-Btr
24	G_{150} 2	+, moderate rod	+, subterminal, oval, +	+	+	-	-	-	+	*Bacillus* sp.	Btr, N-Btr
25	G_{150} 7	+, moderate rod	+, central & subterminal, oval, +	+	+	-	-	-	+	*Bacillus* sp.	Btr, N-Btr

O=Oxidative
F=Fermentative
C=Control
Btr=Bt rhizosphere
N-Bt=Non-Bt
N-Btr=Non-Bt rhizosphere.

Table 41: Results* of estimation of Bt protein (Cry1 Ac) concentration in Control (0 day), Bt, Bt rhizosphere, Non-Bt and Non-Bt rhizosphere (50 and 150 days) soil samples of I crop season, using Envirologix Quantiplate™ Kit for Cry1 Ab/Cry1 Ac.

Blank	1.5 ng*	10 ng*	2.5 ng*
0.000	0.192	0.880	1.727
	0.181	0.898	1.712
	0.171	0.916	1.703

States / Soil Samples	Rajasthan			Gujarat			Maharshtra		
	0 d*	50 d	150 d	0 d	50 d	150 d	0 d	50 d	150 d
Control	-0.005			0.006			-0.038		
Bt		0.019	0.000		0.008	-0.015		-0.024	-0.010
Bt rhizosphere		-0.027	-0.002		-0.008	-0.008		-0.023	-0.010
Non-Bt		-0.008	-0.002		-0.006	-0.003		-0.014	-0.020
Non-Bt rhizosphere		-0.007	0.020		-0.005	0.016		-0.013	-0.010

States / Soil Samples	Karnataka			Andhra Pradesh			Tamil Nadu		
	0 d	50 d	150 d	0 d	50 d	150 d	0 d	50 d	150 d
Control	-0.010			-0.006			0.005		
Bt		-0.003	-0.017		-0.000	-0.007		-0.020	-0.030
Bt rhizosphere		0.026	-0.009		-0.002	-0.007		-0.007	-0.013
Non-Bt		-0.018	-0.007		-0.004	-0.015		0.000	-0.007
Non-Bt rhizosphere		-0.017	0.005		0.002	0.014		0.000	-0.002

*, Values are reading at 450 nm wavelengths in ELISA reader
*, Calibrators available in kit for Cry1 Ac in triplicates
d: Number of days

Table 42: Results* of estimation of Bt protein (Cry1 Ac) concentration in Control (0 day), Bt rhizosphere and Non-Bt rhizosphere (50 and 150 days) soil samples of II crop season, using Cry1 Ac/ Ab Elisa Kit.

Blank	Negative Control*	Positive Control*
0.000	-0.007	F.FFF
	0.003	F.FFF

States / Soil Samples	Rajasthan			Gujarat			Maharshtra		
	0 d	50 d	150 d	0 d	50 d	150 d	0 d	50 d	150 d
Control	-0.010			-0.006			0.016		
Bt rhizosphere		-0.004	0.001		0.002	0.017		-0.008	0.000
Non-Bt rhizosphere		-0.002	-0.010		-0.013	-0.008		-0.001	0.000

States / Soil Samples	Karnataka			Andhra Pradesh			Tamil Nadu		
	0 d	50 d	150 d	0 d	50 d	150 d	0 d	50 d	150 d
Control	0.000			-0.010			0.007		
Bt rhizosphere		-0.005	-0.001		0.016	-0.008		-0.000	-0.009
Non-Bt rhizosphere		0.002	0.003		-0.010	0.019		-0.004	0.000

*, Values are reading at 450 nm wavelengths in ELISA reader
*, Negative Control & Positive Control available in kit for Cry1 Ac
d: Number of days
FFF: Beyond range