
ORIGINAL ARTICLE

Comparative analysis of Antibiotic Susceptibility pattern in
Transgenic Cotton varieties

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ABSTRACT

Transgenic Bt cotton is one of the most adopted transgenic crop throughout the world. Transgenic Bt cotton is considerably effective in controlling lepidopteran pests owing to the presence of Cry genes such as Cry 1Ac (Bollgard I), Cry 1Ac + Cry 2Ab (Bollgard II). In the present study a field trial of Bollgard I and bollgard II of Bt cotton were conducted cyclohexamide and cyclohexamide + kanamycin resistance soil bacteria were isolated from Bt cotton and nBt cotton of Bollgard I and Bollgard II of Bt cotton. NptII gene was isolated from Bollgard I and Bollgard II of Bt cotton. The results were found as cyclohexamide in Bollgard II were found 23.29% higher than Bollgard I of Bt cotton and in the nBt cotton, resistance against cyclohexamide in Bollgard II were found 21.5% higher than Bollgard I of Bt cotton. cyclohexamide + kanamycin in Bollgard I were found 8.9% higher than Bollgard II of Bt cotton and in the nBt cotton, resistance against cyclohexamide + kanamycin in Bollgard I were found 6.5 % higher than Bollgard II of Bt cotton. NptII gene in Bollgard I and Bollgard II of Bt cotton seeds were similarly found.

Key words : Transgenic Bt cotton, Cyclohexamide, Kanamycin, NptII and soil bacteria CFU.

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INTRODUCTION

Transgenic Bt cotton is one of the most adopted transgenic crop throughout the world [1]. Transgenic Bt cotton is considerably effective in controlling lepidopteran pests owing to the presence of Cry genes such as Cry 1Ac (Bollgard I), Cry 1Ac + Cry 2Ab (Bollgard II). Moreover, they are beneficial to the grower and the environment as they reduce chemical insecticides. However, poor performance of the transgenic traits during boll period and variable performance between different regions has been reported [2].

These former bacterial genes could be transferred more easily than other plant genes to soil bacteria because of a high degree of homology facilitating recombination in potential bacterial recipients [3]. Bt-toxin from Bt cotton plants introduced into the soil through two pathways, i.e., biomass bin incorporation and root exudates [4].

Some studies indicate that Bt cotton has no negative effects on soil flora and fauna and may even have beneficial effects [4]. While some studies have reported that Bt cotton creates adverse effects [5]. Similarly Bt cotton contain antibiotic resistance markers such as the neomycin phosphotransferase II (*nptII*) gene which confers resistance to kanamycin and neomycin. The presence of antibiotic resistance genes in transgenic crops has raised concerns over the possible transfer of antibiotic resistance genes from Bt cotton to soil bacteria [6].

The study has been conducted to find out the CFU of (Cyclohexamide and Cyclohexamide + kanamycin) resistance soil bacteria from bollgard I and Bollgard II of soil samples were analyzed and the presence of *nptII* gene were identify in the seed samples of the Bollgard I and Bollgard II of Bt cotton in this experiment.

MATERIAL AND METHODS

Bt cotton seeds such as Bollgard I (cotton 1007- 9810 BG1) and Bollgard II(KCH14K59) Jaddu seeds were obtained from local market, *nptII* primers [7] were obtained from Merck and Chromus, India. The sequence of the primers are as follows *nptII* forward primer 5' CTCACCTTGCTCCTGCCGAGA3'; *nptII* reverse primer 5' CGCCTTGA GCCTGGCGAACAG 3'; Taq DNA polymerase and dNTPs were from Merck. *BL21 DE3*.

Field Trials

The field trails were conducted at the horticulture field and central field of Sam Higginbottom University of Agriculture Technology and Sciences, Allahabad, Uttar Pradesh. Two plots were maintained at a spacing of 0.65m x 0.65m and 0.35 x 0.35m with alternatively sown *Bt* and *nBt* of bollgard I seeds. The selected experimental field was harrowed and ploughed with a cultivator and prepared for sowing. Before sowing the field received one full dose of an organic source of field yard manure (FYM) 1.25 gm/m² were applied in the field. *Bt* cotton and non *Bt*-cotton was be sowed in June 2016. Three plots containing seven rows and seven columns each were maintained at a spacing of 0.65m x 0.65m with 10%, 25% and 50% of refuge *nBt* cotton for Bollgard II, one plot containing eleven rows and seven columns. The recommended agronomic and crop management practices was been followed thereafter. While no pesticide was used during the trial, irrigation was at 10 day intervals initially and before flowering irrigation was done, hand weeding were done one month inter well.

Isolation of Cyclohexamide and Kanamycin Resistant soil bacteria from *Bt* Cotton soil Samples

The soil sample of Bollgard I were collected at 4 and 7 moth after sowing of *Bt* cotton, for bollgard II soil were collected for every two months intervals for three times the crop growing period. Soil samples were collected at 5-7cm depth from the rhizosphere of *Bt* Cotton and *nBt* cotton separately. 2gm of soil was mixed with 15ml PBS and left at room temperature with shaking for 30 minutes in a sterile falcon tube [8]. The mud was removed by centrifugation at 600g for 10 minutes and the supernatant containing bacteria was collected for kanamycin and cyclohexamide screening. 1:10 dilution was plated on cycloheximide + Kanamycin (c+k) and 1:100 cycloheximide (c) LB plates respectively with both cycloheximide and kanamycin at 50 µg/ml. Plates were incubated at 25°C and colonies counted after 1 day for *c* and 2 days for c+k plates and recorded as colony forming units/ 2 mg of soil. When the cLB plates contained a lawn of colonies these plates were not counted while their corresponding c+kLB plates were counted. The plates containing colonies were stored for possible further analysis.

NptII Detection in DNA from Soil Bacteria

NptII detection was done by PCR using specific primers [7]. The PCR reaction was using Taq DNA polymerase with 2.0µl primers, 0.2 µl dNTPs, Taq DNA polymerase 1.0 µl. The reaction condition was 98°C for 10 minutes followed by 40 cycles at 95°C – 30 seconds; 50°C-30 seconds and 72°C – 30 seconds. This was followed by 1 cycle at 72°C for 10 minutes. The positive control for *nptII* gene was *BL21 DE3* containing incorporated cry1AC gene. The PCR product was analyzed on a 2% agarose gel.

Correlation Coefficient Calculation

Phenotypic correlations were estimated using the standard procedure [9] from the corresponding variance and covariance components using the following equation

$$r_{pxy} = \frac{\sigma_{pxy}}{\sqrt{\sigma_{px} \times \sigma_{py}}}$$

Where, r_{pxy} = phenotypic correlation coefficient between characters X and Y ; σ_{pxy} the covariance for X and Y and, σ_{px} and σ_{py} the variance for the two characters X and Y.

CFU/ ml = Number of conilies × Dilution factor / volume of the culture plate

RESULTS AND DISCUSSION

The colony forming unit (CFU) of soil bacteria against cyclohexamide (C) and Cyclohexamide + kanamycin (C+K) of bollgard at 0.65m spacing of Bollgard I *Bt* cotton (Table.1) were found as mean 77.41×10^3 CFU. g^{-2} (C), 19.58×10^2 (C+K). In the spacing of 0.30m the cyclohexamide were found as 70.28×10^3 CFU. g^{-1} (C), were as in cyclohexamide + kanamycin (C+K) were found as 13.3×10^2 CFU. g^{-2} , for the *nBt* cotton were found as mean 40×10^3 CFU. g^{-1} (C), 3.4×10^2 CFU. g^{-2} (C+K). In the spacing of 0.30m the cyclohexamide were found as 52.7×10^3 CFU. g^{-2} (C), were as in cyclohexamide + kanamycin (C+K) were found as 2×10^2 CFU. g^{-2} .

Similarly for the second time colony forming unit (CFU) of soil bacteria of Bollgard I of *Bt* cotton (Table.2) against cyclohexamide (C) and Cyclohexamide + kanamycin (C+K) of bollgard at 0.65m spacing of *Bt* cotton were found as mean 36×10^3 CFU. g^{-2} (C), 10.4×10^2 CFU. g^{-2} (C+K). In the spacing of 0.30m the cyclohexamide were found as mean 45×10^3 CFU. g^{-2} (C), were as in cyclohexamide + kanamycin (C+K) were found as 3, for the *nBt* cotton were found as 77×10^3 CFU. g^{-2} (C), 10×10^2 CFU. g^{-2} (C+K). In the

spacing of 0.30m the cyclohexamide were found as mean 86.4×10^3 CFU. g^{-2} (C), were as in cyclohexamide + kanamycin (C+K) were found as 4.88×10^2 CFU. g^{-2} .

These results were similarly found in *Bt* corn 47×10^6 CFU. g^{-1} . Icoz *et al.* [10] who reported that after 4 consecutive years of corn cultivation. Muchaonyerwa *et al.* [11] reported that antibiotic resistance soil bacteria in *Bt* maize could persist in tropical soils as a result of adsorption on soil clays, but that there were no observable effects on the soil microbial biomass carbon or counts of culturable bacteria. Rui *et al.* [12] found increased numbers of culturable functional groups of bacteria in rhizosphere soil of non *Bt* cotton than in rhizosphere soil of *Bt* cotton in the early and middle stages of growth of cotton. But there was no significant difference on the numbers of these groups.

The colony forming unit(CFU) of *Bt* plants soil bacteria against cyclohexamide (C) of bollgardII (Table.3) at 0.65m spacing of Bollgard II *Bt* cotton at the month of July were found as mean 80×10^3 CFU. g^{-2} in 50% *Bt* with 50% *nBt*, 75%*Bt* with 25%*nBt* and 90%*Bt* with 10%*nBt* similarly and in *Bt* with border refuge of both sides were found as 31.666×10^3 CFU. g^{-2} , were as in C+K the CFU colonies were found as mean 3.666×10^2 CFU. g^{-2} in 50% *Bt* with 50%*nBt*, 287×10^2 CFU. g^{-2} in 75%*Bt* with 25%*nBt*, 48.333×10^2 CFU. g^{-2} and final treatment were found as 49×10^2 CFU. g^{-2} in *Bt* with border refuge of both sides.

Where as in *nBt* cotton the results were found as mean 80×10^3 CFU (Table.3). g^{-2} in 50% *Bt* with 50% *nBt*, 75%*Bt* with 25%*nBt* and 90%*Bt* with 10%*nBt* similarly and in *Bt* with border refuge of both sides were found as 185×10^3 CFU. g^{-2} , were as in C+K the CFU colonies were found as mean 4.666×10^2 CFU. g^{-2} in 50% *Bt* with 50%*nBt*, 204×10^2 CFU. g^{-2} in 75%*Bt* with 25%*nBt*, 276×10^2 CFU. g^{-2} and final treatment were found as 364×10^2 CFU. g^{-2} in *Bt* with border refuge of both sides.

Table. 1 The *Bt* cotton and *nBt* cotton of plant soil samples from the 0.65m and 0.3m on c and c+k plates had a mean colon forming units (CFU) of Bollgard I

Sapcing	<i>Bt</i> cotton				<i>nBt</i> cotton			
	0.65		0.3		0.65		0.3	
	C	C+K	C	C+K	C	C+K	C	C+K
n	75	79	21	21	9	10	5	5
mean	77.41	19.58	70.28	13.3	40	3.4	52.7	2
SD	47.5	31.53	43.4	16.41	17.4	1.82	15.35	1.58
CV (%)	61.4	161	61.75	123.1	42.75	53.5	26.8	79
range	2-208	0-177	35-197	0-45	19-74	1-6	31-70	0-4

Table. 2 The *Bt* cotton and *nBt* cotton of plant soil samples from the 0.65m and 0.3m on cand c+k plates had a mean colon forming units (CFU) of Bollgard I

Sapcing	<i>Bt</i> cotton				<i>nBt</i> cotton			
	0.65		0.3		0.65		0.3	
	C	C+K	C	C+K	C	C+K	C	C+K
n	141	139	36	37	28	28	9	9
mean	36	10.4	45	3	77	10	86.4	4.88
SD	27.8	17.4	59	5.47	42	24.74	27.7	5
CV (%)	77.3	167.7	131	183	54.5	247.4	32	100.8
range	0-164	0-109	3-234	0-30	2-180	0-52	43-128	0-14

The colony forming unit(CFU) of *Bt* plants soil bacteria against cyclohexamide (C) of bollgard at 0.65m spacing of Bollgard II *Bt* cotton at the month of September were found as mean 80×10^3 (Table.4) CFU. g^{-2} in 50% *Bt* with 50% *nBt*, 75%*Bt* with 25%*nBt* and 90%*Bt* with 10%*nBt* were found s 18×10^3 CFU. g^{-2} and in *Bt* with border refuge of both sides were found as 50×10^3 CFU. g^{-2} , were as in C+K the CFU colonies were found as mean 3×10^2 CFU. g^{-2} in 50% *Bt* with 50%*nBt*, 5×10^2 CFU. g^{-2} in 75%*Bt* with 25%*nBt*, 0.333×10^2 CFU. g^{-2} and final treatment were found as 0.666×10^2 CFU. g^{-2} in *Bt* with border refuge of both sides.

Where as in *nBt* cotton the results were found as mean 80×10^3 (Table.4) CFU. g^{-2} in 50% *Bt* with 50% *nBt*, 75%*Bt* with 25%*nBt* and 90%*Bt* with 10%*nBt* were found as 49.666×10^3 CFU. g^{-2} and in *Bt* with border refuge of both sides were found as 21×10^3 CFU. g^{-2} , were as in C+K the CFU colonies were found as mean 26×10^2 CFU. g^{-2} in 50% *Bt* with 50%*nBt*, 58.666×10^2 CFU. g^{-2} in 75%*Bt* with 25%*nBt*, 0.666×10^2 CFU. g^{-2} and final treatment were found as 0.333×10^2 CFU. g^{-2} in *Bt* with border refuge of both sides.

The colony forming unit(CFU) of *Bt* plants soil bacteria against cyclohexamide (C) of bollgard at 0.65m spacing of Bollgard II *Bt* cotton at the month of December were found as mean 53.333×10^3 CFU. g^{-2} in 50% *Bt* with 50% *nBt*, 75%*Bt* with 25%*nBt* were found as 82.666×10^3 (Table.5) CFU. g^{-2} , 90%*Bt* with 10%*nBt* were found s 85.333×10^3 CFU. g^{-2} and in *Bt* with border refuge of both sides were found as 29.333×10^3 CFU. g^{-2} , were as in C+K the CFU colonies were found as mean 10.333×10^2 (Table.5) CFU. g^{-2} in 50% *Bt* with 50%*nBt*, 3×10^2 CFU. g^{-2} in 75%*Bt* with 25%*nBt*, 4.333×10^2 CFU. g^{-2} and final treatment were found as 7.666×10^2 CFU. g^{-2} in *Bt* with border refuge of both sides.

Table 3. The Bt cotton and nBt cotton of plant soil samples from the 0.65m and 0.3m on c and c+k plates had a mean colon forming units (CFU) of Bollgard II (July)

	Bt Plants						nBt Plants								
	C			C+K			C			C+K					
	n	mean	SD	CV(%)	range	n	mean	SD	CV(%)	range	n	mean	SD	CV(%)	range
Bt with border refuge of both sides	3														
50% Bt with 50% nBt	3	31.666		0.00	0	3	3.666		0.00	0	3	185.000		0.00	0
75%Bt with 25% nBt	3	41.868		0.00	0	3	287.333		0.00	0	3	67.352		0.00	0
90%Bt with 10% nBt	3	132.192		0.00	0	3	210.732		0.00	0	3	36.403		0.00	0
50% Bt with 50% nBt	3	73		0	0	3	73.347		0	0	3	125		0	0
75%Bt with 25% nBt	3	3		0	0	3	366		0	0	3	364.000		0	0
90%Bt with 10% nBt	3	3		0	0	3	366		0	0	3	229.188		0	0
50% Bt with 50% nBt	3	49		48.333	3.666	3	3.666		3.666	7	3	364.000		4.666	
75%Bt with 25% nBt	3	78.889		42.525	3.518	3	210.732		210.732		3	204.000		204.000	
90%Bt with 10% nBt	3	160.998		87.987	95.776	3	73.347		73.347		3	204.470		204.470	
50% Bt with 50% nBt	3	140		80	7	3	366		366		3	100.234		100.234	
75%Bt with 25% nBt	3	3		3	3	3	366		366		3	120		120	
90%Bt with 10% nBt	3	3		3	3	3	366		366		3	9		9	

Table 4 The Bt cotton and nBt cotton of plant soil samples from the 0.65m and 0.3m on c and c+k plates had a mean colon forming units (CFU) of Bollgard II (September)

	Bt Plants						nBt Plants								
	C			C+K			C			C+K					
	n	mean	SD	CV(%)	range	n	mean	SD	CV(%)	range	n	mean	SD	CV(%)	range
Bt with border refuge of both sides	3														
50% Bt with 50% nBt	3	18.000		0.00	0	3	3.000		0.00	0	3	21.000		0.00	0
75%Bt with 25% nBt	3	4.585		0.00	0	3	5.000		0.00	0	3	8.881		0.00	0
90%Bt with 10% nBt	3	25.457		0.00	0	3	2.000		0.00	0	3	42.327		0.00	0
50% Bt with 50% nBt	3	50		0	0	3	3.000		0.00	0	3	17		0	0
75%Bt with 25% nBt	3	27.079		0.00	0	3	3.000		0.00	0	3	3		0	0
90%Bt with 10% nBt	3	79.623		25.457	3.666	3	2.647		2.647		3	0.333		0.666	
50% Bt with 50% nBt	3	50		9	3.518	3	2.647		2.647		3	0.666		0.666	
75%Bt with 25% nBt	3	3		3	3	3	40.000		40.000		3	0.573		1.157	
90%Bt with 10% nBt	3	3		3	3	3	40.000		40.000		3	1.157		1.157	
50% Bt with 50% nBt	3	2		1	2	3	5		5		3	173.200		173.200	
75%Bt with 25% nBt	3	3		3	3	3	4		4		3	173.200		173.200	
90%Bt with 10% nBt	3	3		3	3	3	4		4		3	173.200		173.200	
50% Bt with 50% nBt	3	21.000		49.666	80	3	80		80		3	2		2	
75%Bt with 25% nBt	3	8.881		34.580	0.00	3	0.00		0.00		3	64		64	
90%Bt with 10% nBt	3	42.327		69.643	0.00	3	0.00		0.00		3	45		45	
50% Bt with 50% nBt	3	17		68	0	3	0		0		3	45		45	
75%Bt with 25% nBt	3	3		3	3	3	3		3		3	45		45	
90%Bt with 10% nBt	3	3		3	3	3	3		3		3	45		45	

Table. 5 The *Bt* cotton and *nBt* cotton of plant soil samples from the 0.65m and 0.3m on cand c+k plates had a mean colon forming units (CFU) of Bollgard II (December)

	Treatment details																			
	<i>Bt</i> Plants					<i>nBt</i> Plants					C					C+K				
	n	mean	SD	CV(%)	range	n	mean	SD	CV(%)	range	n	mean	SD	CV(%)	range	n	mean	SD	CV(%)	range
50% <i>Bt</i> with 50% <i>nBt</i>	3	53.333	46.180	86.605	80	3	10.333	9.077	87.816	18	3	53.333	46.180	86.605	80	3	10.333	11.842	114.460	21
75% <i>Bt</i> with 25% <i>nBt</i>	3	82.666	4.618	5.582	8	3	3.000	1.730	57.730	3	3	86.000	10.393	12.080	18	3	10.666	6.111	57.281	12
90% <i>Bt</i> with 10% <i>nBt</i>	3	85.333	9.236	10.823	16	3	4.333	2.887	66.613	5	3	80.000	0.000	0.000	00	3	6.000	3.461	57.730	6
<i>Bt</i> with border refuge of both sides.	3	29.333	2.516	8.573	5	3	7.666	7.231	94.358	13	3	80.000	0.000	0.000	00	3	10.000	4.000	40.000	8

Where as in *nBt* cotton the results were found as mean 53.333×10^3 (Table.5) CFU.g⁻² in 50% *Bt* with 50% *nBt* were found as 86×10^3 CFU. g⁻², 75% *Bt* with 25% *nBt*, 90% *Bt* with 10% *nBt* were found as 80×10^3 CFU. g⁻² and in *Bt* with border refuge of both sides were found as 80×10^3 CFU. g⁻², were as in C+K the CFU colonies were found as mean 10.333×10^2 (Table.5) CFU. g⁻² in 50% *Bt* with 50% *nBt*, 10.666×10^2 CFU. g⁻² in 75% *Bt* with 25% *nBt*, 6×10^2 CFU. g⁻² and final treatment were found as 10×10^2 CFU. g⁻² in *Bt* with border refuge of both sides.

These results were similarly found by Pindi and Sultana [13] research results revealed that non *Bt* cotton plant growth is more than *Bt* cotton plant growth and rhizosphere soil sample of non *Bt* cotton has shown increased number of antibiotic. In the studies of Tesfaye *et al.* [14] the CFU were found as 6.3×10^3 CFU. g⁻¹ dry soil in non *Bt* cotton at maturity stage of cotton growth in the field.

Differences in the composition of crop residues as the result of the introduction of transgenic traits have been observed in transgenic *Bt* crops [15]. Other studies have shown that the effects of GM plants on microbial communities depend more on seasonal variations or to other environmental factors, such as soil type and agricultural practices than to expression of Cry or other proteins in plants [16]. The Bollgard I of *Bt* cotton *NptII* results were similarly were found as Singh *et al.* [17].

CONCLUSIONS

The proportion of indigenous soil bacteria resistance against cyclohexamide in Bollgard II were found 23.29% higher than Bollgard I of *Bt* cotton and in the *nBt* cotton, resistance against cyclohexamide in Bollgard II were found 21.5% higher than Bollgard I of *Bt* cotton. The proportion of indigenous soil bacteria resistance against cyclohexamide + kanamycin in Bollgard I were found 8.9% higher than Bollgard II of *Bt* cotton and in the *nBt* cotton, resistance against cyclohexamide + kanamycin in Bollgard I

were found 6.5 % higher than Bollgard II of *Bt* cotton. *NptII* gene in Bollgard I and Bollgard II of *Bt* cotton seeds were similarly found.

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