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Microbial population in soil under transgenic cotton expressing cry proteins from *Bacillus thuringiensis* under irrigated condition

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ABSTRACT: The field experiment was conducted during the *kharif* 2008-2009 and 2009-2010 at Punjab Agricultural University, Ludhiana to study the effect of various plant population and fertilizer levels on seed cotton yield and microflora on sandy loam soil. The results showed that significantly higher seed cotton yield was recorded in *Bt* as compared to non *Bt*. However, the differences in seed cotton yield in different spacings and fertilizer levels found to be non significant. The data showed that there was continual drop of the soil fungal population at 100 DAS and reached the peak at harvest in both *Bt* and non *Bt* cotton during 2008 and 2009. The RCH 134 *Bt* crop recorded the maximum fungal count in soil ($17.8x10^3$ cfu/g) at harvest as compared to the control *i. e.* initial fungal count *i. e.*13.9×10^3 cfu/g before sowing. The fungal count at harvest was better for RCH 134 *Bt* (17.8×10^3 cfu/g) than the RCH 134 non *Bt* (13.42×10^3 cfu/g) during 2008-2009. However, the reverse trend was recorded during 2009-2010. The fungal population dipped at 100 DAS and increased thereafter achieving the highest value at harvest during both the years. The bacterial population showed reduced counts in the soil samples at 70 DAS as compared to the initial counts in both the cotton hybrids during 2008-2009. However, the bacterial count was slightly higher than initial value at 70 DAS during 2009. The bacterial counts of both cotton hybrids consistently increased and reached the highest level either 130 DAS and at harvest during both the years. The cotton varieties RCH 134 *Bt* showed the maximum bacterial counts *i. e.* 21.16×10^7 cfu/g at harvest during the year 2009. The soil actinomycetes counts found less at all the stages of sampling as compared to the counts before sowing *i. e.* 21.8×10^4 cfu/g during 2008-2009. The trends were similar during 2009-2010 too where soil actinomycetes recorded the highest initial counts before sowing (69.7×10^4 cfu/g) and it continued to decline at later stage of the crop. The comparison between *Bt* and non *Bt* cotton crops revealed that higher actinomycetes counts at harvest for RCH 134 *Bt* as compared to non *Bt* during both the years of study.

Key words : Actinomycetes, bacterial count, *Bt* cotton, fungal count, non *Bt*

The challenges of 21st century are to provide food security to the world population and economic stability to the farming community which has pushed agriculture towards cultivation of transgenic crops. These crops are the outcome of biotechnology and liable to pose environmental risks. One such risk is the impact of transgenic plants on the non-target soil micro-flora which depends on the nature of recombinant protein, its release in the soil through root exudates and the extent of exposure. It is an established fact that rhizospheric effect on the soil micro-organisms comes through root exudates which are rich in growth promoting molecules hence microbial population is normally large and active in the

root zone as compared to the bulk soil. Naturally, transgenic plants too release their products like *Bt* toxin and T 4 lysozyme through root exudates and these bind to the soil clay particles and organic components Crecchio and Stotzky (2001); Saxena and Stotzky (2001). The soil bound toxins are reported to retain activity and persist in soil from 140 days to 234 days. The *Bt* cotton and corn plants release *Bt* endotoxin which persists in the soil and retain biological activity. Hence the insect resistant transgenic plants might change their rhizosphere environment through root exudates to promote or retard the activity and multiplication of micro organisms in the rhizosphere. In *Bt* cotton crops, soil persistence of insecticidal cry proteins might have adverse effect on microbial functions and processes (Hu *et al.,* (2009); Icoz *et al.,* (2008)). However, Swilla *et al.,* (2016) reported that single year cultivation of transgenic *Bt* cotton may not affect the functional bacterial and fungal populations in rhizosphere soil. Therefore, the survival, function and diversity of microbial population in the soil is at stake. The present investigation are meant to quantify the microbial populations in the soil under *Bt* cotton *versus* non *Bt* cotton crops at different schedules of cropping as well as agronomic practices so as to assess the impact of *Bt* cotton crops on their numerical status.

MATERIALS AND METHODS

Experimental site : The field experiment was conducted during the *kharif* 2008-2009 and 2009-2010 at Punjab Agricultural University, Ludhiana to study the effect of various plant population and fertilizer levels on the *Bt* toxin content in leaves of *Bt* cotton. The experimental site (30° 56'N, 75° 52'E; 247 m ASL), was having deep alluvial sandy loam in texture, slightly alkaline or near-neutral in pH (7.5), low in organic carbon (0.31), medium in available phosphorus (12.5 kg/ha) and high in available potash (281 kg/ha). The weather parameters at the experimental site were given in Table 1.

Field experiment : The experiment was laid out in double split plot design with 2 cotton hybrids *i. e.* RCH 134 *Bt* and RCH 134 non *Bt* in main plots, 2 spacings *i. e.* 67.5 x 90 cm and 100 x 75 cm in sub plots and 3 fertilizer levels *viz.,* recommended dose of fertilizer (RDF) (150:30:30 NPK kg/ha), 125 per cent of RDF (187.5:37.5:37.5 NPK kg/ha) and 150 per cent of RDF (225 : 45 : 45 NPK kg/ha) in sub sub plots with 3 replications. The soil of the experimental field was sandy loam in texture, neutral in pH (7.2-7.5), low in organic carbon (0.22 to 0.31), medium in available phosphorus (12.5-17.7 kg/ha) and high in available potash (281-380 kg/ha). A heavy pre sowing irrigation (10 cm) was applied for fine seed bed preparation. The experiment was sown on 7 May and 10 May during 2008 and 2009, respectively by dibbling method. No P and K fertilizers were applied as the phosphorus and potash status of the experimental site was medium and high respectively. Nitrogen was applied @ 150 kg/ha. Half the nitrogen was applied at the time of first irrigation and remaining half at the time of flowering in the month of July. All the cultural practices regarding weed control, irrigation and insect pest management were followed as per Punjab Agricultural University recommendations. The soil samples from various

Table 1. Weather parameters in crop season during *Kharif* 2008 and 2009

Standard week	Maximum temperature (^{0}C)		Minimum temperature (^{0}C)		Rainfall (mm)	
	2008	2009	2008	2009	2008	2009
14	27.2	31.8	15.6	16.5	50.2	25.0
15	33.9	33.7	18.7	16.4	0.0	0.0
16	35.4	36.7	16.4	19.5	0.0	0.0
17	39.5	38.1	19.5	17.9	0.0	0.0
18	40.3	37.5	21.8	21.5	4.2	6.4
19	37.5	37.3	23.5	20.7	0.2	0.0
20	35.4	41.5	24.3	25.5	56.8	0.0
21	32.2	40.5	22.3	24.5	6.0	0.0
22	37.7	39.1	23.4	25.6	0.0	0.0
23	36.2	41.6	26.5	25.0	6.4	0.0
24	33.3	37.7	26.6	24.2	80.0	6.0
25	33.9	40.8	24.5	24.6	90.6	0.0
26	32.9	39.9	24.8	26.5	100.3	106.6
27	34.1	36.4	28.1	26.5	15.8	0.0
28	33.7	33.2	26.3	26.7	38.7	61.9
29	34.4	34.8	26.7	26.9	55.6	102.6
30	34.8	31.3	28.4	26.1	39.8	309.6
31	32.3	34.3	26.7	27.2	61.2	23.0
32	32.6	36.0	26.3	28.7	108.8	0.0
33	31.3	33.4	24.8	27.3	176.4	24.8
34	34.3	33.6	26.8	25.5	6.2	35.2
35	33.3	32.9	25.1	24.9	49.0	50.2
36	32.5	31.4	24.9	23.3	24.9	7.5
37	33.9	30.5	24.4	22.4	0.0	62.4
38	29.5	34.3	20.9	24.3	19.8	0.0
39	33.2	34.7	21.9	24.7	0.0	0.0
40	33.6	32.9	23.8	22.3	19.8	26.2
41	33.3	32.8	21.0	18.5	0.0	0.0
42	31.6	31.7	16.7	15.3	19.2	0.0
43	30.5	30.3	16.7	11.3	0.0	0.0
44	30.4	30.3	15.2	13.6	0.0	0.0
45	29.6	26.3	12.6	12.5	0.0	1.0
46	27.5	23.8	12.8	11.8	0.0	4.1
47	24.9	23.7	8.9	7.1	0.4	0.0

treatments were collected from different treatments for estimation of various micro flora present in the soil at the time of sowing, 70, 100, 130 DAS and at the time of harvest of the crop.

Soil microbial studies : The composite soil samples were taken at 0 (before sowing), 70, 100 and 130 DAS and at harvest. Four samples of each treatment were taken at 0-15 cm depth and mixed so as to have a representative sample. The viable microbial populations were analyzed by the standard technique of Serial Dilution and Pour Plating. The details of culture media used

and technique followed are as follows [1].

i) Composition of culture media (/1)

a) Soil Extract Agar : Used for bacteria

Glucose	:	1.0 g
KH_2SO_4	:	0.5 g
KNO_3	:	0.1 g
Soil extract	:	100 ml
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	6.8-7.0

*1 kg fertile soil with 1 g $CaCO_3$ powder, litre water, boiled for 1 h and the extract decanted

b) Dextrose Nitrate Agar : Used for actinomycetes

Glucose	:	1.0 g
KH_2SO_4	:	0.1 g
KCl	:	0.1 g
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	7.0-7.2

c) Rose Bengal Agar :Used for fungi (exclusively moulds)

Dextrose	:	10 g
Peptone	:	5.0 g
KH_2PO_4	:	1.0 g
$MgSO_4.7H_2O$	:	0.5 g
Rose Bengal	:	0.033 G
Agar	:	15.0 g
Distilled water	:	1000 ml
pH	:	5.5

The media were prepared and sterilized in an autoclave using super heated steam at 15 psi pressure for 20 min. The sterilized media flasks were used immediately or kept for future use.

ii) Preparation of dilution blanks: Distilled water solution of NaCl, 0.85 per cent was prepared and dispensed in test tubes, 9 ml each. These dilution blanks were cotton plugged and sterilized in an autoclave as described above. These were used after cooling or kept for future use.

iii) Serial dilution : The representative soil samples sealed in polybags were brought to the laboratory, opened under aseptic conditions and approximately 1 g sample was transferred to a pre-sterilized, pre-weighed sample bottle and weighed again. These were serially diluted *i. e.* 10/dilution at each step using dilution blanks until desired dilution of the samples (upto 10^{-7}) were obtained.

iv) Pour Plating : Pre sterilized polypropylene or glass petri dishes were used for plating of diluted soil samples in triplicate. Using sterilized pipettes, 1 ml of the diluted sample was transferred to a petri dish followed by pouring melted agar gel (at about 50° C) @ 20-25 ml/ petri

Table 2 Effect of nutrient levels and plant geometry on yield of *Bt* and non *Bt* cotton

Treatments	Seed cotton yield (kg/ha)	
	Ist Year 2008-2009	IInd year 2009-2010
Hybrids		
RCH134 *Bt*	3340.9	2403.8
RCH134 Non *Bt*	2277.0	1837.0
LSD (p=0.05)	640.1	99.6
Spacing (cm)		
67.5X90	2805.5	2089.4
100X75	2812.2	2151.4
LSD (p=0.05)	NS	NS
Fertilizer level (kg/ha) RDF*	2832.1	2085.6
(125%) RDF	2770.6	2094.8
(150%) RDF	2824.0	2180.9
LSD (p=0.05)	NS	NS
Interaction	NS	NS

*RDF: Recommended dose of fertilizers (150:30:30 NPK kg/ha)

dish. The dish was gently rotated immediately after pouring to mix the contents and allowed to cool and solidify.

v) Incubation and colony counting : The poured petri dishes for bacteria and actinomycetes were incubated at 30+1°C in an inverted position for 5-7 days until the countable colonies of each type developed. The respective colonies were counted by visual observation based on their characteristics as follows:

Fungi : Showing mycelia cottony growth on the agar surface with or without variously coloured spores.

Actinomycetes : Showing white, dull white or grey coloured small size colonies with powdery surface.

Bacteria : Slimy wet or partially wet, minute pin head to large spreading colonies on the agar surface.

The plates which showed colony counts between 30-300 were selected. The microbial counts were expressed as colony forming units/g (cfu/g) after making necessary calculations of means and unit weights of a sample.

RESULTS AND DISCUSSION

Seed cotton yield : The results showed that significantly higher seed cotton yield was recorded in *Bt* as compared to non *Bt* (Table 2). However, the differences in seed cotton yield in different spacings and fertilizer levels found to be non significant. The higher seed cotton yield of *Bt* cotton as compared to non *Bt* due to less pest attack was also reported by Buttar and Singh (2006).

Fungal count : The continual drop of the soil fungal population upto 100 DAS, then rise at 130 DAS and reaching the peak at harvest for both *Bt* and non *Bt* cotton crops was observed in the year 2008 (Table 3). The RCH 134 *Bt* cotton crop recorded the maximum fungal count, $17.8x10^3$ cfu/g soil at harvest as compared to the control *i. e.* initial fungal count, 13.9×10^3 cfu/g before sowing. The fungal count at harvest was better for RCH 134 *Bt* (17.8×10^3 cfu/g) than the RCH 134 non *Bt* (13.42×10^3 cfu/g). During the year 2009 too, the fungal population dipped upto 100 DAS and increased thereafter achieving the highest value at harvest, however, the RCH 134 non *Bt* was better at 19.4×10^3 cfu/g than RCH 134 *Bt* at 17.3×10^3 cfu/g both being higher than the initial fungal count before sowing, 12.7×10^3 cfu/g (Table 4). The continual drop of the soil fungal population upto 100 DAS, then rise at 130 DAS and reaching the peak at harvest for both *Bt* and non *Bt* cotton crops was observed in the year 2008-2009 (Table 3). Such variation in the microbial populations were also reported in several field studies conducted by Icoz and Stotzky (2008a), Hu *et al.,* (2009) and Wang *et al.,* (2009) .

Bacterial population : The bacterial population showed reduced counts in the soil samples at 70 DAS when compared to the initial counts before sowing in both the cotton varieties in the year 2008-2009 (Table 3) but the results in 2009-2010 were varied at this stage of sampling (Table 4). In the later stages, however, the bacterial counts of these cotton varieties consistently increased to reach the highest values at either 130 DAS or at harvest for both the years. The cotton varieties RCH 134 *Bt* and non *Bt* showed the maximum bacterial counts, 21.16×10^7 cfu/g at harvest and 12.08×10^7 cfu/

Table 3 Effect of nutrient levels and plant geometry on soil micro flora of *Bt* and non *Bt* cotton (Ist year)

Treatment	Fungi (cfu*/g fresh wt.of soil				Bacteria (cfu/g fresh wt.of soil)				Actinomycetes (cfu/g fresh wt.of soil)			
	70DAS	100DAS	130DAS	At harvest	70DAS	100DAS	130DAS	At harvest	70DAS	100DAS	130DAS	At harvest
Hybrids												
RCH134 *Bt*	4.15x10	0.22x10	7.02x10	17.18x10	1.03x10	3.72x10	13.28x10	$10.58x10^7$	8.90x10	$7.40x10^4$	$12.82x10^4$	$19.61x10^4$
RCH134 Non *Bt*	5.58x10	0.48x10	7.35x10	13.42x10	1.08x10	4.57x10	**12.0x10⁷8**	$12.50x10^7$	8.97x10	$7.55x10^4$	$12.08x10^4$	$14.50x10^4$
Spacing (cm)												
67.5x90	4.83x10	0.40x10	8.12x10	14.55x10	1.11x10	4.13x10	9.80x10	$10.63x10^7$	9.25x10	$8.7x10^4$	$13.58x10^4$	$15.73x10^4$
100x75	4.9x10	0.30x10	6.25x10	16.05x10	1.01x10	4.15x10	15.57x10	12.45v	8.62x10	$6.22x10^4$	$11.32x10^4$	$18.38x10^4$
Fertilizer level (kg/ha)	5.58x10	0.28x10	7.30x10	17.23x10	1.17x10	3.70x10	13.7x10 ⁷5	11.35x10	9.5x10	$6.82x10^4$	$13.93x10^4$	$18.05x10^4$
RDF												
(125%) RDF	3.9x10	0.38x10	6.88x10	20.90x10	**.077x10**	3.70x10	$12.9x10^75$	10.78x10	8.0x10	$6.88x10^4$	$12.48x10^4$	$17.18x10^4$
(150%) RDF	5.05x10	0.40x10	7.38x10	7.78x10	1.25x10	5.03x10	$11.3x10^78$	12.50x10	9.23x10	$8.73x10^4$	$10.95x10^4$	$15.95x10^4$

*cfu – Colony forming unit

Initial Microflora before sowing : Fungi – 13.9 x 10^3, Bacteria – 2.94 x 10^7, Actinomycetes – 21.8 x 10^4

Table 4 Effect of nutrient levels and plant geometry on soil micro flora of *Bt* and non *Bt* cotton (IInd year)

Treatment	Fungi (cfu*/g fresh wt.of soil				Bacteria (cfu/g fresh wt.of soil)				Actinomycetes (cfu/g fresh wt.of soil)			
	70DAS	100DAS	130DAS	At harvest	70DAS	100DAS	130DAS	At harvest	70DAS	100DAS	130DAS	At harvest
Hybrids												
RCH134 *Bt*	$3.92x10^3$	$1.51x10^3$	$8.95x10^3$	$17.3x10^3$	$3.72x10^7$	$8.71x10^7$	$14.05x10^7$	$21.16 x10^7$	$68.37x10^4$	$24.55 x10^4$	$9.01 x10^4$	$10.36 x10^4$
RCH134 Non *Bt*	$5.34x10^3$	$4.25x10^3$	$4.15x10^3$	$19.4x10^3$	$3.93x10^7$	$7.76x10^7$	$12.08x10^7$	$11.55 x10^7$	$61.44 x10^4$	$42.29 x10^4$	$5.74 x10^4$	$9.24 x10^4$
Spacing (cm)												
67.5x90	$4.85x10^3$	$2.03x10^3$	$7.81x10^3$	$18.22x10^3$	$3.24x10^7$	$8.92x10^7$	$13.21 x10^7$	$19.91 x10^7$	$63.61 x10^4$	$35.36 x10^4$	$7.40 x10^4$	$9.07 x10^4$
100x75	$4.41x10^3$	$3.73x10^3$	$5.30x10^3$	$18.46x10^3$	$4.42x10^7$	$7.55x10^7$	$12.92 x10^7$	$12.79 x10^7$	$66.21 x10^4$	$31.28 x10^4$	$7.36 x10^4$	$6.53 x10^4$
Fertilizer level (kg/ha)	$4.40x10^3$	$2.68x10^3$	$6.06x10^3$	$16.96x10^3$	$4.16x10^7$	$9.47x10^7$	$14.03 x10^75$	$14.65 x10^7$	$69.96 x10^4$	$40.48 x10^4$	$7.93 x10^4$	$6.29 x10^4$
RDF												
(125%) RDF	$5.58x10^3$	$2.74x10^3$	$6.72x10^3$	$21.04x10^3$	$3.37x10^7$	$6.19x10^7$	$12.26 x10^73$	$16.39 x10^7$	$61.29 x10^4$	$31.28 x10^4$	$7.92 x10^4$	$8.28 x10^4$
(150%) RDF	$3.91x10^3$	$3.22x10^3$	$6.89x10^3$	$17.00x10^3$	$3.76x10^7$	$9.05x10^7$	$12.92 x10^78$	$18.02 x10^7$	$63.47 x10^4$	$26.63 x10^4$	$6.29 x10^4$	$8.83 x10^4$

*cfu/g soil

Initial Microflora before sowing: Fungi – 12.7 x 10^3, Bacteria – 3.01 x 10^7, Actinomycetes – 69.7 x 10^4

g respectively in the year 2009. The increasing population of bacteria with increase in age indicates the stimulation effect of root exudates of both *Bt* and non *Bt* plants. Since *Bt* plants results in higher bacterial population than that in non *Bt* plants, it appears *Bt* proteins were the source of additional growth promoting factors as reported by Shen *et al.,* (2006).

Actinomycetes : The soil actinomycetes counts reported lower at all stages of sampling *i. e.* 70, 100 and 130 DAS and at harvest when compared to the counts before sowing 21.8 x 10^4 cfu/g in 2008 (Table 3). The trends were similar in 2009-2010 too where soil actinomycetes recorded highest initial counts before sowing 69.7 x 10^4 cfu/g and it continued to decline at different DAS, lowest being at 130 DAS and harvest (Table 4). The comparison between *Bt* and non *Bt* cotton crops revealed higher actinomycetes counts at harvest for RCH 134 *Bt* Vs RCH 134 non *Bt i. e.* 19.61 x 10+4 Vs 14.5 x 104 cfu/g in 2008 and 10.36 x 104 V/s 9.24 x 10^4 cfu/g in 2009-2010. The root exudates of *Bt* and non *Bt* plants did not contribute towards growth stimulation of actinomycetes , hence their population declined progressively at different DAS. Furthermore no significance could be attached to the role of *Bt* root exudates for decline of actinomycetes population *v/s* non *Bt* exudates. Exceptionally lower counts of actinomycetes in soil samples of *Bt* cotton (24.55 x 10^{4+} cfu/g) in comparison to non *Bt* cotton (42.29 x 10^4) cfu/g) at 100 DAS during the year 2009-2010 may be attributed to the inhibitory effect of *Bt* toxin which might have accumulated in the rhizosphere and degraded later on as reported by Icoz and Stotzky (2008b).

CONCLUSION

The data showed that there was continual drop of the soil fungal population upto 100 DAS, rise at 130 DAS and reaching the peak at harvest for both *Bt* and non *Bt* cotton crops. The RCH 134 *Bt* cotton crop recorded the maximum fungal count in soil (17.8x10^3 cfu/g) at harvest as compared to the control *i. e.* initial fungal count *i. e.*13.9 x 10^3 cfu/g before sowing. The fungal count at harvest was better for RCH 134 *Bt* (17.8 x 10^3 cfu/g) than the RCH 134 non *Bt* (13.42 x 10^3 cfu/g) during 2008-2009. The fungal population dipped at 100 DAS and increased thereafter achieving the highest value at harvest during 2009-2010. However, the RCH 134 non *Bt* showed better count than RCH 134 *Bt* and both showed higher fungal count than before sowing. The bacterial population showed reduced counts in the soil samples at 70 DAS as compared to the initial counts in both the cotton hybrids during 2008-2009. However, the results during 2009-2010 were varied at this stage of sampling. The bacterial counts of both cotton hybrids consistently increased and reached the highest level either 130 DAS or at harvest during both the years. The cotton varieties RCH 134 *Bt* and non *Bt* showed the maximum bacterial counts *i. e.* 21.16 x 10^7 cfu/g and 12.08 x 10^7 cfu/g at harvest, respectively during the year 2009-2010. The soil actinomycetes counts found less at all stages of sampling as compared to the counts before sowing *i. e.* 21.8 x 10^4 cfu/g during 2008. The trends were similar during 2009-2010 too where soil actinomycetes recorded the highest initial counts before sowing (69.7 x 10^4 cfu/g) and it continued to decline at later stage

of the crop. The comparison between *Bt* and non *Bt* cotton crops revealed that higher actinomycetes counts at harvest for RCH 134 *Bt* as compared to non *Bt* during both the years of study.

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