J. Biol. Control, 22(2): 357-367, 2008

Enhanced persistence of insecticidal crystal proteins of *Bacillus thuringiensis* subsp *kurstaki* by transforming the predominant phyllosphere bacterium, *Bacillus megaterium*

R. ASOKAN*, K. S. MOHAN# and VAGEESHBABU S. HANUR

Division of Biotechnology,
Indian Institute of Horticultural Research,
Hessaraghatta Lake (PO), Bangalore 560 089, Karnataka, India.
E-mail: asokan@iihr.ernet.in

ABSTRACT: **The insecticidal crystal proteins (ICP) of *Bacillus thuringiensis* (Bt) have relatively short persistence when applied as foliar spray. The limited persistence of the ICP on the phyllosphere is mainly due to alkaline pH and UV radiation in the solar spectrum. In this regard, chemical adjutants, plant derived substances such as phenols and flavinoids have been employed to improve the persistence of the ICP. Alternatively, expression of ICP in a predominant phyllosphere bacterium has been shown to be useful in enhancing the persistence of the same. Therefore, *Bacillus megaterium*, a predominant phyllosphere bacterium of cabbage, was engineered to express the ICP of Bt subsp *kurstaki* (Btk) through conjugal transfer. A total of seven stable transformants, *viz.*, A3, B3, C3, E1, E2, E3 & F were obtained in the above. Among them, B3 was highly toxic to the important pests of cabbage, *Plutella xylostella* (0.36 ng/cm^2) and *Crocidolomia binotalis* (1.74 ng/cm^2). Additionally, B3 had extended lysis (96 hours) and higher persistence (9 cfu/cm^2) as compared to the rest of the transformants and Btk.**

KEY WORDS: *Bacillus thuringiensis* subsp *kurstaki*, *B. megaterium*, conjugation, enhanced persistence

INTRODUCTION

Bacillus thuringiensis (Bt) is a ubiquitous, gram positive soil bacterium that produces insecticidal crystal proteins (ICP) during sporulation. The various Bt formulations are successfully used in agriculture and public health with no adverse effect on non-target organisms including humans. The main factor limiting the field efficacy and frequency of application of Bt is the relative short persistence of ICP (about two days after application) due to various factors (Bora *et al.*, 1994). Persistence of ICP is mainly limited by leaf pH and UV rays, particularly UV-B (280-320 nm) & UV-A (320-380 nm) in the solar spectrum (Sudarsan *et al.*, 1994). Therefore, in order to increase the persistence, jaggery, chemical whiteners, plant secondary metabolites like phenols, flavinoids, etc. have been used in the tank mix (Jacobs and Sundin, 2001). Alternatively,

* Corresponding author

Monsanto Research Centre, # 44/2A,Vasanth's Business Park, Bellary Road, Bangalore – 560 092, India

engineering the predominant phyllosphere bacterium of cabbage to express the ICP is a viable approach (Bora *et al.*, 1994; Downing *et al.*, 2000). Therefore, the predominant phyllosphere bacterium, *B. megaterium* of cabbage was transformed to express the ICP of Btk through conjugal transfer. Among the various transformants, the promising one was selected based on the toxicity and extended lysis and persistence on phyllosphere of cabbage. This approach is cost-effective and needs less time than developing transgenic plants (Fcitelson *et al.*, 1992).

MATERIALS AND METHODS

a) Conjugation of Btk and *B. megaterium*

In a previous study, screening of Btk and *B. megaterium* with different antibiotics showed that the former was susceptible to nalidixic acid (Nal) at 7.5μg/ml and rifampsin (Rif) at 10μg/ml, while the later was resistant. In the present investigation the above antibiotics were used in order to facilitate the selection of tranformants.

Conjugation of Btk and *B. megaterium* was carried out according to Bora *et al.* (1994). The *log* phase cultures of Btk and *B. megaterium* were mixed in 5:1 ratio and were spotted on sterile nitrocellulose paper which was placed on LB agar and incubated at 30°C, overnight. The putative transformants were selected by spreading 100 μl of the serial dilutions (10^{-1} to 10^{-4}) of the above conjugation mix on LB agar containing Nal and Rif (7.5 and 10μg/ml, respectively) and incubated at 30°C for 72 hours. Smears of colonies that appeared on the antibiotic plates were heat fixed, stained with crystal violet and observed under oil immersion (100 x) in a light microscope for the production of ICP. All the colonies that produced ICP were further purified by single colony isolation and maintained on nutrient agar at 4°C until further use.

b) Microbiological and molecular characterization of the transformants

Prior to microbiological and molecular characterization, stability of ICP production of the various putative transformants was checked by 20 continuous subculturing in LB agar. The transformants were further characterized by (i) colony morphology (ii) visual estimation on the extent of lysis and (iii) sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) (SDS-PAGE) (Laemmli, 1970).

For comparing the colony morphology, the appearance of various transformants was compared with that of Btk and *B. megaterium*. For visual estimation on the extent of lysis, the various transformants, Btk and *B. megaterium* were grown in LB agar and incubated at 30°C for 72 hours. Smears from the above cultures were heat fixed, stained with crystal violet and observed under oil immersion (100 x) in a light microscope at 24 hours interval for 96 hours. For SDS-PAGE, spore-crystal mixtures of the transformants, Btk and *B. megaterium* were digested in 2 N NaOH for one hour at room temperature and centrifuged at 8000 rpm at five minutes. An aliquot of the supernatant was used for protein estimation using Lowry's method (Lowry *et al.*, 1951) and 30 μl of each culture was used for SDS-PAGE. Protein profiles were observed in a gel documentation system after staining the gel with Coomassie brilliant blue-R250.

c) Bioassay

Stock culture of test insects, *viz. Plutella xylostella* and *Crocidolomia binotalis* were maintained on cabbage plants at the Division of Biotechnology, Indian Institute of Horticultural Research, Bangalore.

The various transformants, Btk and *B. megaterium* were grown on LB agar at 30°C for 72 hours. The cells were harvested using a sterile plastic cell scrapper, homogenized on a magnetic stirrer in sterile distilled water and this suspension formed the crude-crystal preparation (CCP). An aliquot from the CCP from the above cultures was used for protein estimation using Lowry's method (Lowry *et al.*, 1951). Serial dilutions of the CCP were prepared in sterile distilled water and 200 μl of different dilutions were applied on both sides (@ 100μl/side) of fresh cabbage leaf discs ($62cm^2$). Single treated leaf disc was placed in individual sterile plastic Petri plate and 20 numbers of five-day-old larvae of *P. xylostella* and *C. binotalis* were released separately. There were five replications for

each treatment and an untreated control was also maintained. Mortality of the treated larvae was recorded at 24 hours interval and subjected to probit analysis (Abbott, 1925) and toxicity was expressed in ng/cm^2.

d) Persistence of the transformant on the phyllosphere of cabbage

The promising transformant that exhibited higher toxicity and extended lysis was selected for carrying out studies on persistence on the phyllosphere of cabbage. The most promising transformant, Btk and *B. megaterium* were grown on LB broth at 30°C for 72 hours. 200 µl of 1 OD concentration of the above cultures were smeared @ 100µl/side on both surfaces of 10 leaves of potted cabbage plants. Treated leaves were identified by sticking a red colour tape on the petiole. There were five replications per treatment and an untreated control was also maintained. Observations on the persistence of the above cultures were made at 24 hours interval for the first week after inoculation and later at weekly interval for three weeks. A single treated leaf was selected randomly, washed in 100 ml sterile distilled water, and centrifuged at 8000 rpm at room temperature for 10 minutes. The pellet was resuspended in 100 µl of sterile distilled water and plated on LB agar containing Nal and Rif (7.5 and 10 µg/ml, respectively) and incubated at 30°C for 72 hours. To determine the persistence of Btk and *B. megaterium*, the pellet was plated on LB agar without antibiotics. Smears from the above cultures were heat fixed, stained with crystal violet and observed under oil immersion (100 x) in a light microscope for the production of ICP. Population of each culture was expressed as colony forming units per centimeter square (cfu/cm^2). The data was subjected to analysis of variance (ANOVA).

RESULTS AND DISCUSSION

a) Conjugation and characterization of transformants

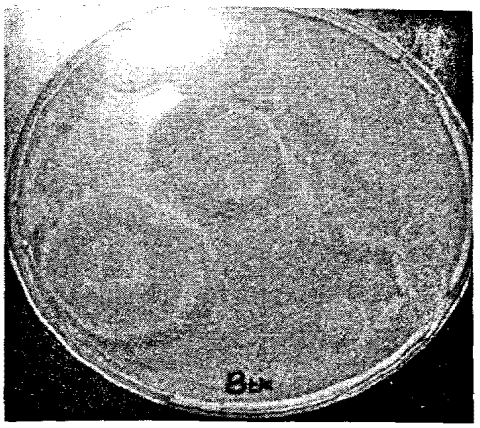
Conjugation of Btk and *B. megaterium* yielded seven putative transformants, *viz.*, B3, C3, E1, E2, E3 and F. The colony morphology of the seven transformants was the same as that of *B. megaterium* (Fig.1). Light microscopic observations of the various transformants showed that all the transformants produced bipyramidal ICP (Fig.2). Therefore, it was clear that expression of ICP did not alter the colony morphology of the transformants indicating that the characteristics of *B. megaterium* were intact and that could have facilitated the selection of the transformants in antibiotic medium. The various transformants produced bigger ICP as compared to Btk. The average size of the ICP produced by the various transformants were 1.8 x 3.9 µm (range - 1.6 to 2.1 x 3.0 to 4.6 µm) as compared to Btk (1.0 x 2.3µm, range - 0.9 to 1.3 x 1.5 to 3.0µm). Bigger size of the ICP produced by the transformants could be attributed to the relatively larger size of *B. megaterium* cells as compared to Btk (Fig.2). Observations on the lysis pattern showed that the transformant B3 had extended lysis and complete lysis was observed after 96 hours which was similarly observed in *B. megaterium* (Table 1). McGuire and Shasha (1990) recorded that a variety of environmental factors such as sunlight, temperature, rainfall, and UV radiation affect the field efficacy of microbial insecticides. Therefore, the extended lysis that was observed in the transformant B3 could be advantageous to improve the field persistence of the ICP by not directly exposing the same to the UV radiation. SDS-PAGE profile showed that all the transformants had 140 kDa and 66 kDa bands as observed in Btk (Fig.3). This also confirmed the stable transformation and expression of ICP in various transformants.

b) Bioassay

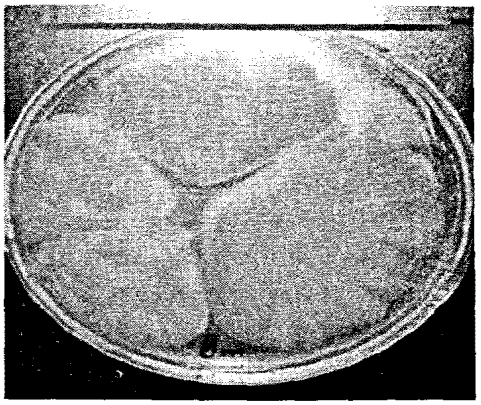
Among the seven transformants, B3 was found to be highly toxic to both *P. xylostella* (0.36 ng/cm^2) and *C. binotalis* (1.74ng/cm^2). The difference in LC_{50} value for *P. xylostella* and *C. binotalis* was 0.59, 1.38, 7.25, 3.62, 2.65, 6.74, 1.45 and 2.17 ng/cm^2 for A3, B3, C3, E1, E2, E3, F and Btk, respectively. There was no mortality in *B. megaterium* treatment and untreated control. Comparison of LC_{50} values for the transformant B3 and Btk showed a variation of 0.63 and 1.42 ng/cm^2 for *P. xylostella* and *C. binotalis*, respectively (Table 2, 3). There were two transformants, *viz.*, A3

Table 1. Lysis pattern of various transformants, Btk and *B. megaterium* (*Bm*)

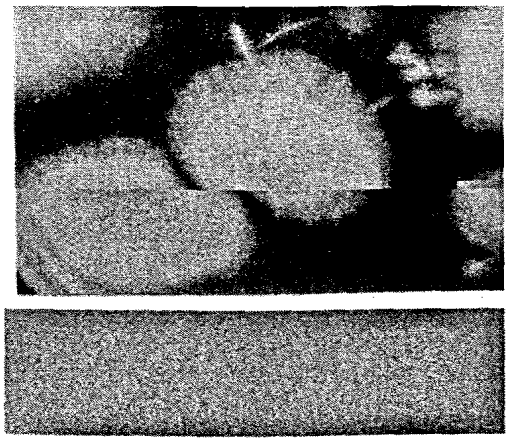
Culture	24 hours			48 hours			72 hours			96 hours		
	Sporulation	crystal	Lysis	Sporulation	crystal	Lysis	Sporulation	crystal	Lysis	Sporulation	crystal	Lysis
A3	40%	Not visible	No lysis	100%	Inside the cell	70%	***	Mostly outside the cell	100%	***	***	***
B3	40%	Not visible	No lysis	100%	Inside the cell	40%	***	Inside & outside the cell	70%	***	Comply. outside thecell	100%
C3	40%	Not visible	No lysis	100%	Inside the cell	70%	***	mostly outside the cell	100%	***	***	***
E1	50%	Not visible	No lysis	100%	Inside the cell	70%	***	mostly outside the cell	100%	***	***	***
E2	50%	Not visible	No lysis	100%	Inside the cell	70%	***	mostly outside the cell	100%	***	***	***
E3	60%	Not visible	No lysis	100%	Inside the cell	70%	***	mostly outside the cell	100%	***	***	***
F	70%	Not visible	No lysis	100%	Inside the cell	70%	***	mostly outside the cell	100%	***	***	***
Btk	90%	Inside the cell	No lysis	100%	Inside & out side the cell	>75%	***	Outside the cell	100%	NA	NA	NA
Bm	50%	***	No lysis	100%	***	40%	***	***	75%	***	***	100%



⇨ *Bacillus thuringiensis* subsp *kurstaki*

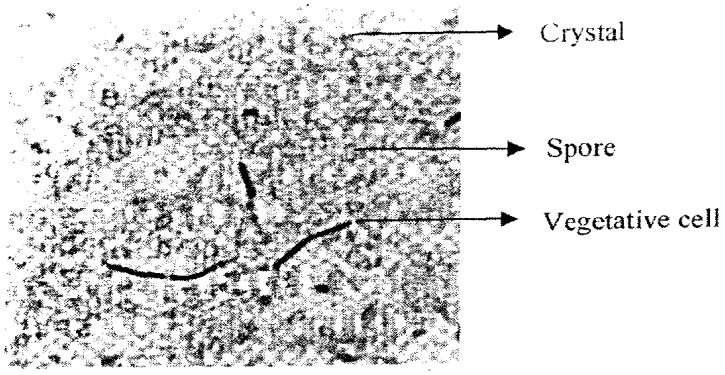


⇨ *Bacillus megaterium*

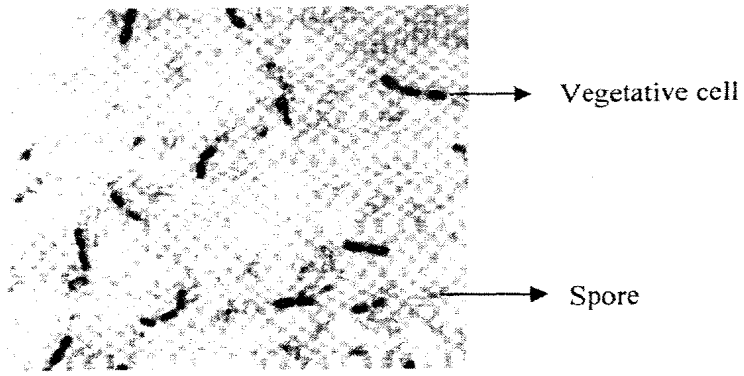


⇨ Transformant B3

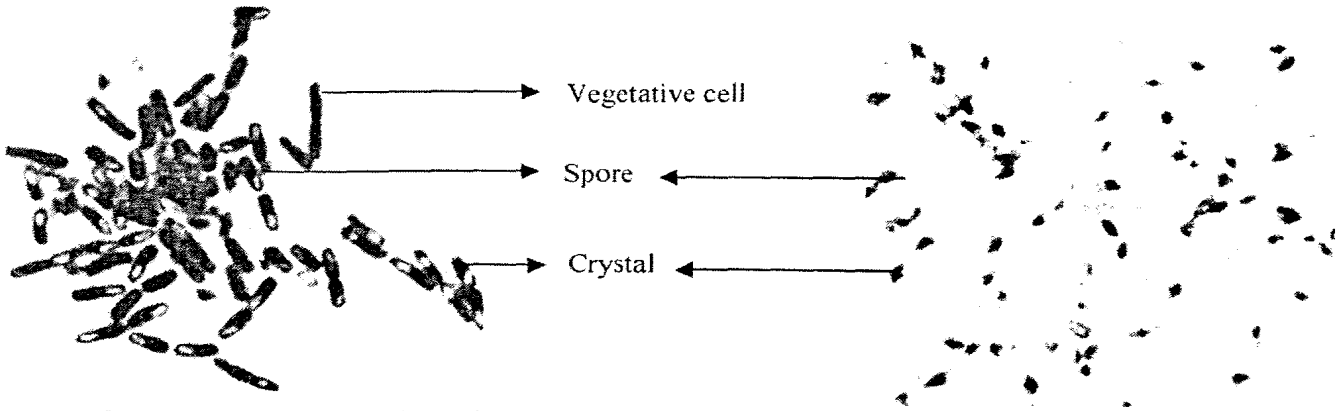
Figure 1. Colony morphology of Btk, *B. megaterium* and the transformant B3



Bacillus thuringiensis subsp *kurstaki*



Bacillus megaterium (untransformed)



Transformant B3, sporulation phase

Transformant B3, lysed phase

Fig 2. Light microscopic images of various stages of Btk, *B. megaterium* and the transformant B3

and F that recorded higher toxicity against *P. xylsotella* as compared to Btk. Similarly, three transformants, *viz.*, C3, E1 and E2 had higher toxicity against *C. binotalis* as compared to Btk (Table 2 & 3). One of the reasons for higher toxicity of the transformant B3 could be due to the large size of crystals and also due to differences in the protoxin composition of the crystals as compared to the other transformants and Btk. Similarly, Bora *et al.* (1994) observed that RS-1, a transformant of *B. megaterium*, was highly toxic to the larvae of *Helicoverpa armigera* as compared to Btk.

c) Persistence of the transformant B3 on phyllosphere of cabbage

Studies on the persistence of the transformant B3, Btk and *B. megaterium* showed that the B3 was recorded for up to 15 days after inoculation (9 cfu/cm^2), whereas Btk was recorded only for up to 3 days after inoculation (2.67 cfu/cm^2). The population level of *B. megaterium* was high and almost consistent throughout the period of experiment where it ranged from 132.33 to 174. 33 cfu/cm^2. The reduction in the population level during the second day after application was 38 and 155.67 cfu/cm^2 for B3 and Btk, respectively, as compared to *B. megaterium*. A difference of 92. 33, 95.7, 118.3, 93.63, 118.72 and 132.7 cfu/cm^2 was observed between the transformant B3 and *B. megaterium* during 3rd, 4th, 5th, 6th, 7th and 15th days after inoculation whereas a huge difference of 156.33 and 171.66 cfu/cm^2 was observed between Btk and *B. megaterium* during 2nd and 3rd day of inoculation. Similarly a difference of 117.67 and 79.33 cfu/cm^2 was observed between the transformant B3 and Btk during 2nd and 3rd day after inoculation (Table-4).

While selecting a suitable candidate phyllosphere bacterium for conjugal transfer, it is important to select one that belongs to the same genus of Bt. Since Btk and *B. megaterium* belonged to Bacillaceae, conjugal transfer could have been successful. Similarly expression of *cry*2A of Bt in a related bacterium like *B. cereus* resulted in high level of expression (Moar *et al.*, 1994). Contrary to the above, expression of ICP of Bt in *Pseudomonas cepacia* that belongs to Pseudomonadaceae resulted in poor expression (Stock *et al.*, 1990). Similarly when the mosquitocidal toxin gene *cry*4B of Bt was expressed in the cyanobacterium, *Agmenellum quadruplicatum*, there was very low level of expression (Downing *et al.*, 2000). The increased persistence of the transformant B3 as compared to Btk on the phyllosphere of cabbage could be due to the fresh production of insecticidal crystals by the surviving transformed cells through repeated cycles of vegetative and sporulation stages. The other factors such as leaf pH and germinant condition of the cabbage leaves would have been suitable for the transformant B3 as against Btk (Caipo *et al.*, 2002). Even though competitive interaction between the transformant B3 and *B. megaterium* was apparent, persistence of B3 was higher (15 days after inoculation) than that of Btk (three days after inoculation) (Table 4). Bora *et al.* (1994) and Sudarsan *et al.* (1994) reported that establishment of the microbial population depends on the leaf morphology and presence of recognition factors. Therefore, the phyllospheric competence of the transformant B3 is partly similar to that of *B. megaterium* so it could survive for a longer time as compared to Btk. In addition to the above factors, inter-specific competition and compositions among the different microbial communities on the phyllosphere determine the adaptation of the transformants on the leaf (Beattie and Lindow, 1999; Lindow and Leveau, 2002).

The stability and quantity of the insecticidal crystals of Bt on the leaf surface are the two most limiting factors in obtaining satisfactory control of insect pests. In this regard, Bt formulations containing various encapsulating agents did not prevent the loss of sprays due to rain (Bora *et al.*, 1994). Therefore, ICP produced in a leaf colonizing bacteria is a practicable alternative in improving the persistence on the phyllosphere as observed by Moar *et al.* (1994). Hence the transformant B3 could be mass produced to facilitate successful management of *P. xylostella* and *C. binotalis* and other lepidopterans on cabbage.

Table 2. Toxicity of various transformants to *Crocidolomia binotalis*

Sl No.	Cultures	LC_{50} (ng/cm^2)	Slope (±SE)	95% Fiducial Limit	
				Lower	Upper
1	A3	2.01	1.93 (±0.16)	1.69	2.33
2	B3	1.74	0.16 (±0.01)	1.72	1.76
3	C3	7.76	1.41 (±0.07)	7.62	7.90
4	E1	4.24	1.94 (±0.16)	3.92	4.56
5	E2	3.16	2.56 (±0.28)	2.60	3.72
6	E3	7.74	0.86 (±0.07)	7.60	7.88
7	F	2.80	2.33 (±0.24)	2.32	3.28
8	Bt subsp *kurstaki*	3.16	2.11 (±0.19)	2.78	3.54
9	*B. megaterium*	0.00	0.00 (±0.00)	-	-
10	Control	0.00	0.00 (±0.00)	-	-

Table 3. Toxicity of various transformants to *Plutella xylostella*

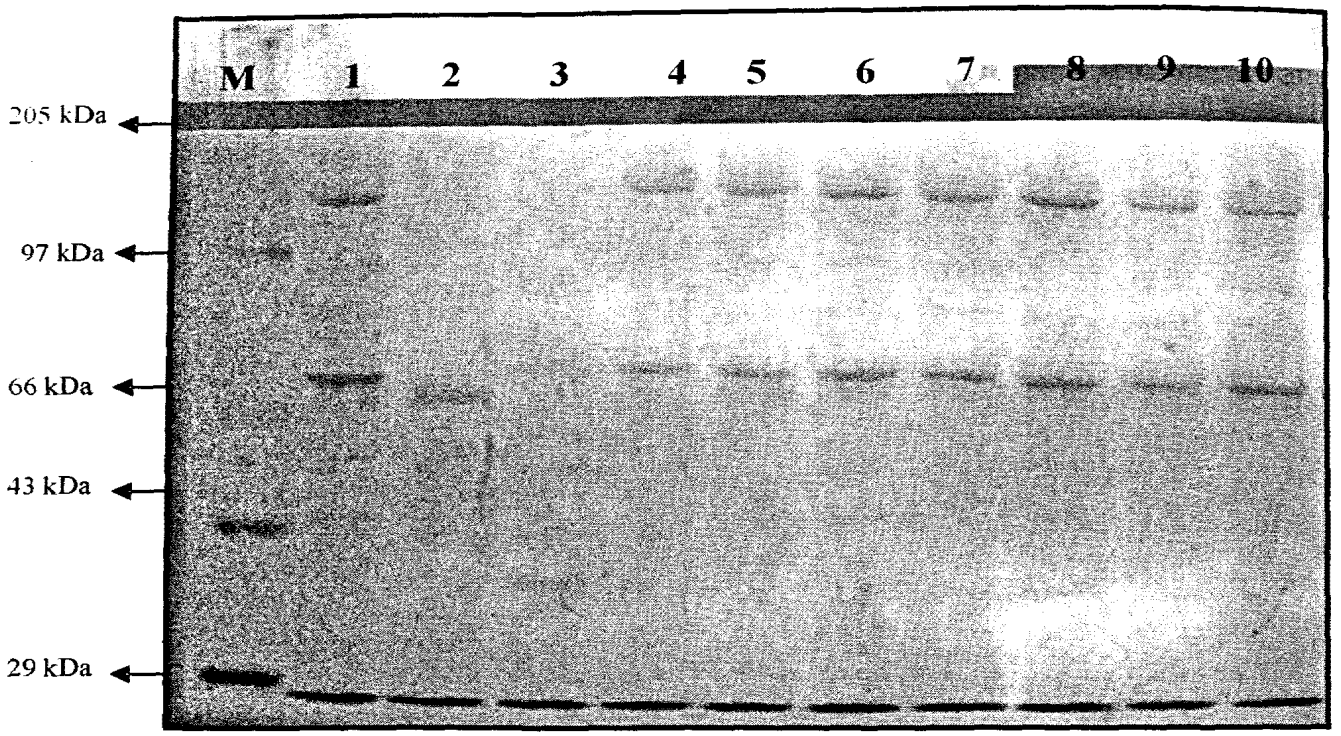
Sl No.	Cultures	LC_{50} (ng/cm^2)	Slope (±SE)	95% Fiducial Limit	
				Lower	Upper
1	A3	1.42	0.91 (±0.08)	1.26	1.58
2	B3	0.36	0.86 (±0.01)	0.34	0.38
3	C3	0.51	0.86 (±0.07)	0.37	0.75
4	E1	0.62	1.17 (±0.02)	0.58	0.66
5	E2	0.51	0.86 (±0.01)	0.37	0.75
6	E3	1.00	0.96 (±0.01)	0.98	1.02
7	F	1.35	0.99 (±0.01)	1.33	1.37
8	Bt subsp *kurstaki*	0.99	0.96 (±0.01)	0.97	1.01
9	*B. megaterium*	0.00	0.00 ± 0.00)	-	-
10	Control	0.00	0.00 ± 0.00)	-	-

Table 4. Persistence of the transformant B3 as compared to Btk and *B. megaterium* on phyllosphere of cabbage

Culture	Population (cfu/cm^2)										Mean
	2 DAI	3 DAI	4 DAI	5 DAI	6 DAI	7 DAI	15 DAI	21 DAI	28 DAI	35 DAI	
Transformant B3	127.00 (11.29)	82.00 (9.08)	64.00 (8.03)	45.70 (6.79)	38.70 (6.26)	27.00 (5.24)	9.00 (3.08)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	39.34 (5.19^b)
Btk	9.33 (3.14)	2.67 (1.77)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	0.00 (0.71)	1.20 (1.05^c)
B. megaterium	165.00 (12.86)	174.33 (13.22)	159.7 (12.66)	164.00 (12.83)	132.33 (11.53)	145.72 (12.09)	141.70 (11.92)	157.00 (12.55)	176.70 (13.31)	158.00 (12.59)	157.45 (12.55^a)

DAI – Days after inoculation, SEM – 0.41 P<0.01 CD = 2.771

Figures in parentheses are square root transformed values



Legend: M- Marker, Lane 1 - Btk, Lane 2 - Btk (HD-1), Lane 3 - *B. megaterium*, Lanes 4 to 10 – transformants A3, B3, C3, E1, E2, E3, and F

Fig 3. Protein profile of Btk, *B. megaterium* and various transformants

ACKNOWLEDGEMENTS

The authors are grateful to the Director, Indian Institute of Horticultural Research (IIHR), Bangalore, for encouragement and facilities and Dr. Venugopalan, IIHR, Bangalore, for statistical analysis.

REFERENCES

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, **18**: 265-267.

Beattie, G. A. and Lindow, S. E. 1999. Bacterial colonization of leaves: A spectrum of strategies. *Phytopathology*, **89**: 353–359.

Bora, R. S., Murty, M. G., Shenbagarathai, R. and Sekar, V. 1994. Introduction of a lepidopteran-specific insecticidal crystal protein gene of *Bacillus thuringiensis* subsp *kurstaki* by conjugal transfer into a *Bacillus megaterium* strain that persists in the cotton phyllosphere. *Applied and Environmental Microbiology*, **60**: 214-222.

Caipo, M. L., Duffy, S., Zhao, L. And Schaffner, D. W. 2002. *Bacillus megaterium* spore germination is influenced by inoculum size. *Journal of Applied Microbiology*, **92**: 879-884.

Downing, K. J., Leslie, G. and Thomson, J. A. 2000. Biocontrol of the sugarcane borer, *Eldana saccharina* by expression of the *Bacillus thuringiensis cry*1Ac7 and *Serratia marcescens chi* genes in sugarcane associated bacteria. *Applied and Environmental Microbiology*, **66**: 2804–2810.

Feitelson, J. S., Payne, J. and Kim, L. 1992. *Bacillus thuringiensis*: insects and beyond. *Bio/Technology*, **10**: 271–275.

Jacobs, J. L. and Sundin, G. W. 2001. Effect of UV-B

radiation on a phyllosphere bacterial community. *Applied and Environmental Microbiology*, **67**: 5488-5496.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.

Lindow, S. E. and Leveau, H. J. 2002. Phyllosphere microbiology. *Current Opinion in Biotechnology*, **13**: 1-5.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin-Phenol reagents. *Journal of Biological Chemistry*, **193**: 265-275.

McGuire, M. R. and Shasha, B. S. 1990. Sprayable self encapsulating starch formulations of *Bacillus thuringiensis*. *Journal of Economic Entomology*, **83**: 1813–1817.

Moar, W. J., Trumble, J. T., Hice, R. H. and Backman, P. A. 1994. Insecticidal activity of the CryIIA protein from the NRD-12 isolate of *Bacillus thuringiensis* subsp *kurstaki* expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a leaf colonizing strain of *Bacillus cereus*. *Applied and Environmental Microbiology*, **60**: 896-902.

Stock, C. A., McLoughlin, T. J., Klein, J. A. and Adang, M. J. 1990. Expression of a *Bacillus thuringiensis* crystal protein gene in *Pseudomonas cepacia* 526. *Canadian Journal of Microbiology*, **36**: 879–884.

Sudarsan, N., Suma, N. R., Vennison, S. J. and Sekar, V. 1994. Survival of a strain of *Bacillus megaterium* carrying a lepidopteran-specific gene of *Bacillus thuringiensis* in the phyllospheres of various economically important plants. *Plant and Soil*, **167**: 321–324.

(Received: 26.10.2007; Revised: 18.12.2007; Accepted: 25.02.2008)