RESEARCH PAPER Asian Journal of Bio Science, Vol. 4 Issue 2 : 278-285 (October, 2009 to March, 2010)

Characterization of native *Bacillus thuringiensis* from the Western Ghats of Kerala

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(Accepted : September, 2009)

Eleven *Bacillus thuringiensis* isolates were obtained from the soils of Western Ghat region of Kerala. Morphology of the isolates on LBA media revealed similarity with *Bacillus thuringiensis* HDI. All the isolates produced creamy white, puffy colonies. Staining with Coomassie brilliant blue revealed the presence of dark blue crystal proteins having spherical, irregular, bipyramidal and triangular shapes. Biochemical characterization of the isolates showed variable response to hydrolysis of urea, starch and esculin. Profiling of cry1 and cry4 genes from *B. thuringienis* isolates was done using universal cry1 and cry4 primers for detection of cry genes and prediction of their insecticidal activities. Amplification with universal cry1 gene primer was obtained for eight isolates KY2, KY3, KY5, KY6, KK7 KY8, KY9 and EM11 along with the reference strain HD1. Presence of cry 4 gene, specific to dipteran larvae was detected in isolates KY1 and EM10.

Key words : *Bacillus thuringiensis*, ecological niche, parasporal inclusions, biochemical tests

INTRODUCTION

The continous and prolonged use of synthetic pesticides has led to the emergence of resistance in agricultural pests and to environmental degradation. This has created an urgent need for the development of environment friendly pesticides to reduce contamination and the likelihood of insect resistance (Sheltan *et al.*, 2002). Recently, there has been a renewed interest in the development of biological alternatives to chemical pesticides.

Bacillus thuringiensis Berliner is considered as one of the most versatile microbial insecticides. It has been used as a successful biological insecticide for more than 40 years and is a uniquely specific, safe and effective tool for the control of a wide variety of insect pests (Nester *et al.*, 2002). The use of *Bacillus thuringiensis* as a microbial insecticide has several advantages over the use of chemical control agents, as they are highly specific for certain hosts and are not toxic to other insects, plants and vertebrates.

Bacillus thuringiensis is a rod shaped, gram positive, facultative anaerobic, spore forming bacterium. The insecticidal activity is based on the ability of the bacterium to produce large quantities of larvicidal proteins known as delta-endotoxins. These delta-endotoxins are toxic to more than 150 insects belonging to the orders Lepiodptera, Diptera, Coleoptera and many others.

As a result of the increased selection pressure and continuous exposure to a single kind of toxin, insect pests have evolved varying levels of resistance. In order to face the certainity of wide spread *Bacillus thuringiensis* resistance, wise management strategies have to be adopted. Intensive screening programmes are going on worldwide to isolate large number of *B. thuringiensis*, in order to identify new strains with increased levels of insecticidal activity against a broader spectrum of insect pests. The natural habitat of *Bacillus thuringiensis* are plant surfaces, trees, soils, stored products and insect hosts. It is a wide spread bactrium detected in different habitats including soil, grain dust, diseased insect larvae, deciduous and coniferous leaves and sericultural farms (Dulmage and Aizawa,1982, Obeidat *et al.*, 2004). Soil is the natural reservoir for insecticidal *Bacillus thuringiensis* with moderate to low toxicities (Ishi and Ohba, 1993).

In the present study, *B. thuringiensis* isolates were obtained from the Western Ghats of Kerala. The Western Ghats of India is one among the eighteen hotspots of biodiversity of the world, which is relatively undisturbed and is expected to harbour novel isolates of *B. thuringiensis*. Morphological and biochemical characterization of the isolates was also done.

MATERIALS AND METHODS

The experiment was carried out in the Molecular Biology Laboratory of the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Thrissur, Kerala during the period 2005-2007. The chemicals used for the study were obtained from different

agencies including MERCK, SRL and HIMEDIA, Bangalore. The chemicals used were of analytical grade. The Taq DNA polymerase, 10X assay buffer and the needed dNTPs were obtained from M/s. Bangalore Genei Pvt. Ltd. The primers were synthesized from Integrated DNA Technologies, USA. Soil samples were collected from the Western Ghats of Kerala, coming under three districts namely Kottayam, Kozhikode and Ernakulam. Samples were taken from the top 5 to 10cm soil in polythene covers. The location, altitude and latitude were recorded with the help of Geographical positioning System (GPS Model Etrex, Garmin, USA).

Bacillus thuringiensis was isolated from soil samples collected from Western Ghats of Kerala according to the procedure described by Travers *et al.* (1987). Initially 0.5 g of soil sample was added to test tubes containing 9ml of Luria-Bertani (LB) broth buffered with 0.25 M sodium acetate. The broth was incubated for four hours at 30°C with shaking on an incubator at 200 rpm. Aliquots of 1ml were transferred to sterilized test tubes and heated to 80°C for 3 minutes. 1ml from this was transferred to a 9ml sterile water blank to get 10^{-1} dilution. This was mixed thoroughly and ten fold dilutions were made upto 10^{-3}. 100ml each taken from 10^{-2} and 10^{-3} dilutions were pour plated on LB agar medium. The plates were then incubated at 28 ± 2°C for 24-48 hours. The colonies formed were picked up based on their morphological similarities with standard *Bacillus thuringiensis* colonies. The selected colonies were purified by repeated streaking on T3 agar medium until pure single colonies were obtained. The cultures were maintained as stabs in cryostorage vials in LB agar medium and stored under refrigerated conditions.

The bacterial isolates were characterized using staining reactions like Gram, endospore and crystal protein staining. The reference strain HD1 (Kronstad and Whitely, 1986) was used in all the studies. The isolates were subjected to Gram staining reaction following the procedure of Hucker and Conn (1923). Endospore staining was done according to procedure developed by Smirnoff (1962). The cultures were examined for the presence of crystal protein inclusions by staining with Coomassie brilliant blue as described by Shariff and Alaeddinoglu (1983).

Bacillus thuringiensis isolates were characterized using seven different biochemical tests namely response to KOH, hydrolysis of urea, gelatin, starch, lecithin, esculin and Voges Proskauer test. All the biochemical tests were carried out following Cappuccino and Sherman (1992).

The genomic DNA of *B. thuringiensis* isolates used for PCR amplification was isolated following the procedure of Sambrook and Russel (2001). The PCR was carried out by using 25ng of DNA template in a reaction mixture(total volume 25µl) containing each deoxynucleoside triphosphate at a concentration of 10mM, each primer at a concentration of 8pM and 0.3U of Taq DNA polymerase dissolved in corresponding reaction buffer (Promega). Amplifications were performed in a Eppendorf Master Cycler, Gradient (Eppendorf, Germany) under the following conditions, 2 minutes of initial denaturation at 94°C, followed by 30 cycles of denaturation for 45 seconds at 94°C, annealing for 1 minute at 55°C depending on DNA template and extension for 2 minutes at 72°C. An extra extension step consisting of 10 minutes at 72°C was added after completion of 35 cycles. PCR products were resolved on 0.8 percent agarose gel stained with ethidium bromide and located with ë DNA/Eco R1+ Hind111 double digest as a DNA molecular weight marker. The DNA bands in gels were documented using gel documentation system (Alpha imager TM1200).

All the isolates were screened for the presence of cryI and cry4 genes using universal primers designed by Ben-Dov *et al.* (1997). Total DNA was isolated from *B. thuringiensis* strains obtained from the Western Ghats of Kerala, in order to amplify the cry genes. A set of universal primers Un1(d), Un1(r), Un4(d) and Un4(r) (Ben-Dov *et al.*, 1997) were used for the amplification of cryI and cry4 genes in *Bacillus thuringiensis*. The details of the primers used for the study are given in Table 1.

Table 1: Details of primers used for the experiment

Sr. No.	Primer	Primer sequence*	Length (bp)	Tm (°C)
1.	Un1(d)	5' CATGATTCATGCGGCAGATAAAC 3'	23	54. 7
2.	Un1(r)	5' TTGTGACACTTCTGCTTCCCATT 3'	23	57
3.	Un4(d)	5' GCATATGATGTAGCGAAACAAGCC 3'	24	56.6
4.	Un4(r)	5' GCGTGACATACCCATTCCAGGTCC 3'	25	61.6

* Source- Ben-Dov *et al.*, 1997

RESULTS AND DISCUSSION

Eleven soil samples were collected from the Western Ghat regions of Kerala, coming under three districts namely Kottayam, Kozhikode and Ernakulam (Fig.1). The details of the soil samples collected and *Bacillus thuringiensis* isolated are given in Table 2. The isolates were named based on the districts from where it was isolated. A total of 30 spore forming bacteria were obtained, out of which 19 were *B. thuringiensis* isolates. Soil samples collected from wild ecosystems of Western Ghats of Kerala, yielded a total of 19 B.thuringiensis isolates, by sodium acetate selection method. The Western Ghats is an area of exceptional biodiversity and possesses an undisturbed ecological niche. Great biodiversity among *B. thuringiensis* isolates collected from this area is due to its co-evolution along with the insects or by conjugal transfer of plasmid to other strains. Earlier workers have collected samples from different geographical locations such as forests, agricultural lands, industrial areas and plant surfaces. Intensive screening programmes identified bacterial strains from these sources (De Lucca *et al.*, 1991; Chilcott and Wigley, 1993;

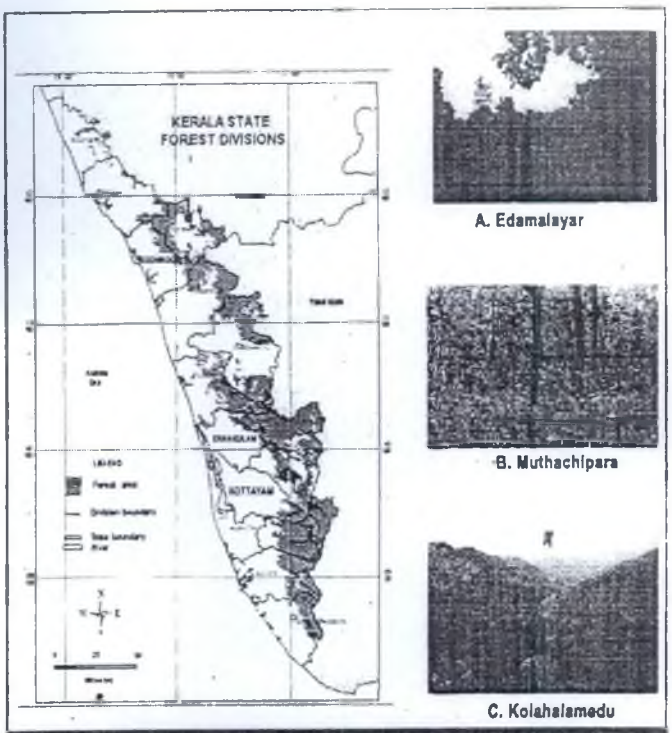

Fig 1. : Western Ghats of Kerala showing the districts surveyed for soil sample collection

Table 2 : Details of *Bacillus thuringiensis* isolates obtained from soil samples from the Western Ghats

Sr. No.	District and location	No. of colony forming units (cfu)/ 0.5 g soil $\times 10^2$			*B.thuringiensis* selected	GPS values	
		Total no.	Spore formers	*B. thuringiensis*		Altitude	Elevation (ft)
1.	Kottayam-Paruthumpara	Many	3	2	KY1	N:9°38.252 E:76°56.510	3210
2.	"	"	3	2	KY2	N:9°38.250 E:76°56.510	3210
3.	"	"	1	1	KY3	N:9°34.125 E:76°32.310	3278
4.	Kottayam-Kolahalamedu	"	5	3	KY4	N:9°38.520 E:76°55.134	3606
5.	"	"	2	1	KY5	N:9°38.210 E:76°55.175	3230
6.	"	"	2	1	KY6	N:9°35.531 E:76°52.213	3532
7.	Kozhikode-Muthachipara	"	3	1	KK7	N:11°31.489 E:75°49.557	3401
8.	Kozhikode-Thumbithullumpara	"	2	1	KK8	N:11°28.432 E:75°3.678	2255
9.	Kozhikode-Thusharagiri	"	1	1	KK9	N:10°28.402 E:76°3.554	1813
10.	Ernakulam-Edamalayar	"	3	1	EM10	N:10°12.556 E:76°42.318	989
11.	"	"	5	5	EM11	N:10°13.212 E:76°43.554	1012
	Total		30	19			

Benhard *et al.*, 1997; Valicente and Barreto, 2003).

In the present study, sodium acetate selection method was followed for isolating *B. thuringiensis* from soil samples. Placing the soil samples in growth media and incubating them allowed the maximum germination of unwanted spores. Subsequent heat treatment eliminated the excess contaminating organisms. Sodium acetate inhibited the germination of *B. thuringiensis* spores, so that other spores germinated. The soil samples were analysed for total spore counts and occurrence of *Bacillus thuringiensis*. Similar method was adopted by earlier scientists (Leithy *et al.*, 2004, Carrozi *et al.*, 1991). The isolates produced creamy white puffy large colonies, typical of *Bacillus thuringiensis* when grown on LB agar medium.

The isolates of *B. thuringiensis* from different locations showed slight variations in cultural characteristics. All the isolates produced creamy white, puffy colonies 24 hours after inoculation on LB agar medium. Except for the isolates KY3, KK8 and EM11, which produced medium sized colonies, all other isolates produced large sized colonies. Form of the colonies varied from circular to irregular, with flat elevation. Margin of the colonies varied from entire to undulate. Observations made on the colony characteristics of *Bacillus thuringiensis* isolates are given in Table 3. Similar variations in the morphological characteristics of *B. thuringiensis* isolates on nutrient agar medium were recorded by Chatterjee *et al.* (2006).

All isolates were found to have blue colour after Gram staining indicating Gram positive nature and the cells were rod-shaped. Endospore staining of the isolates showed the presence of spores as translucent and the crystals as bluish black in colour. Staining of the isolates with Coomassie brilliant blue revealed the presence of dark bluish crystal proteins. The shape of crystal proteins varied from irregular, spherical, triangular and bipyramidal for different isolates. Isolates KY1, KY5 and KK7 produced a composite of spherical and bipyramidal crystal proteins. Triangular and spherical crystal proteins were observed in KK8. Isolates KK9 and KY4 produced irregular crystal proteins. Of the total 11 isolates analyzed, eight produced spherical crystal proteins (Table 4 and Fig 2). Composite parasporal inclusion bodies have been

Table 3 : Colony characteristics of native isolates of *Bacillus thuringiensis*

Sr. No.	Isolate	Size	Form	Margin	Diameter (mm)
1.	KY1	Large	Circular	Entire	6 .0
2.	KY2	Very large	Circular	Entire	9.0
3.	KY3	Medium	Circular	Entire	4.0
4.	KY4	Large	Irregular	Undulate	5.0
5.	KY5	Large	Circular	Undulate	120
6.	KY6	Large	Circular	Entire	5.0
7.	KK7	Large	Circular	Entire	6.0
8.	KK8	Medium	Circular	Entire	4.0
9	KK9	Large	Circular	Undulate	7.0
10.	EM10	Large	Irregular	Undulate	5.0
11.	EM11	Large	Circular	Entire	6.0
12.	HD1	Large	Circular	Entire	7.0

Table 4 : Crystal protein morphology of native isolates of *Bacillus thuringiensis* and the reference strain HD1

Sr. No.	Isolate	Shape of crystal protein
1.	KY1	Spherical and bipyramidal
2.	KY2	Spherical
3.	KY3	Spherical
4.	KY4	Irregular
5.	KY5	Spherical and bipyramidal
6.	KY6	Spherical
7.	KK7	Spherical and bipyramidal
8.	KK8	Triangular and spherical
9.	KK9	Irregular
10.	EM10	Spherical
11.	EM11	Spherical
12.	HD1	Bipyramidal

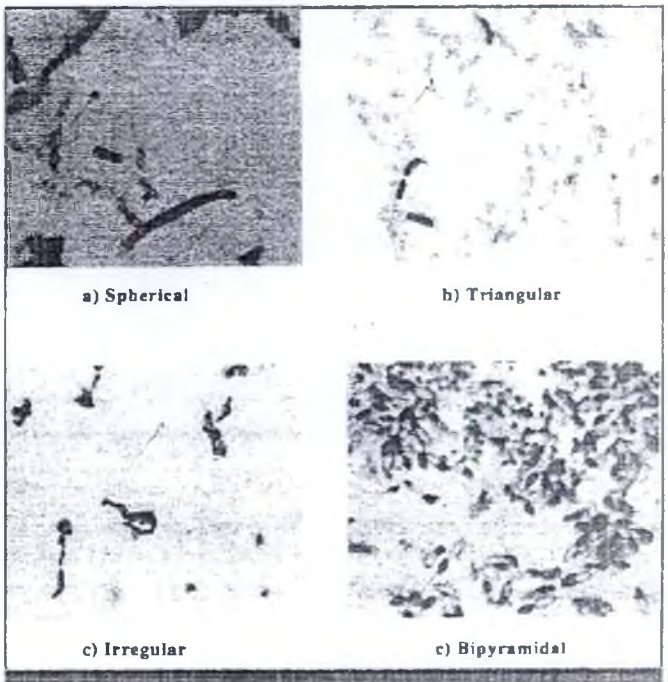

Fig 2 : Crystal protein morphology of native isolates of *B. thuringiensis*

earlier reported by Obeidat *et al.* (2004). The parasporal inclusion bodies of *Bacillus thuringiensis* were observed as morphologically heterogeneous (Ohba *et al.*, 2001). Benhard *et al.* (1997) observed crystal protein morphology of *Bacillus thuringiensis* isolates as diverse ranging from spherical, bipyramidal, rectangular, irregularly spherical and irregular pointed.

Biochemical characterization of the isolates was done using seven different biochemical tests. Details of the biochemical characterization are given in Table 5. Testing the solubility of bacterial cells in three per cent of KOH is an effective supplement to Gram staining, proven to be useful for rapid and accurate differentiation of bacteria (Suslow *et al.*, 1982). None of the isolates formed viscous thread-like structure with KOH, indicating gram positive reaction.

In gelatin hydrolysis test, after 24 hours incubation, gelatin remained soldified indicating that the isolates were not capable of producing proteolytic extra-cellular enzyme gelatinase. Negative reaction of bacterial isolates to gelatin hydrolysis was reported by earlier workers (Hernandez *et al.*, 1998; Demir *et al.*, 2002).

Nine isolates namely KY1, KY5, KY6, KK7, KK8, KK9, EM11 along with HD1 were found to degrade urea. This was indicative of presence of urease enzyme. The remaining isolates KY3, KY4 and EM 10 gave negative results for urease test. Differential response of bacterial isolates to hydrolysis of urea had been reported by earlier workers (Hernandez *et al.*, 1998; Demir *et al.*, 2002; Chatterjee *et al.*, 2006; Sharmin and Rahmin, 2007).

Except for the isolate KK9, all other isolates including HD1 gave a positive reaction, indicating the presence of amylase, the starch splitting enzyme. Similar observations were made by Demir *et al.* (2002) who characterized *B. thuringiensis* isolates using starch hydrolysis reaction and all gave positive response.

All the isolates were negative for Voges Proskauer test indicating the absence of non-acidic/neutral end products such as acetyl methyl carbinol from glucose metabolism. Reports on negative reaction of bacterial isolates to Voges- Proskauer test were made by various scientists (Hernandez *et al.*, 1988; Demir *et al.*, 2002; Lopez and Alippi, 2005).

All the isolates gave positive reaction for lecithinase test. A clear zone was observed around the growth of bacteria which indicated a positive reaction. Hydrolysis of lecithin by bacterial strains isolated from diverse locations was reported by various scientists (Hernandez *et al.*, 1989; Demir *et al.*, 2002).

Isolates KY1, KY2, KY4, KY5, KY6, and EM11along with HD1 hydrolyzed esculin which was indicated by a blackening around the growth of bacterium. Reports on bacterial isolates that differ in their reaction to esculinase were made by various scientists (Gordon *et al.*, 1973; De Lucca *et al.*, 1981; Hernandez *et al.*, 1998).

Among the different biochemical tests used for the characterization of *B. thuringiensis* isolates, differential response was obtained for urea hydrolysis, starch hydrolysis and esculinase test. The response of the bacterial isolates to three per cent KOH, gelatin hydrolysis, Voges-Proskauer test and lecithinase test remained uniform. The present study provides an understanding of the diversity of *B. thuringiensis* strains in the habitats of Western Ghat region of Kerala. The differential response of bacterial isolates to biochemical tests is indicative of the diversity in locations from which *B. thuringiensis*

Table 5. Biochemical characteristics of *Bacillus thuringiensis* isolates obtained from soil samples from the Western Ghats

Sr. No.	Isolate	Biochemical test						
		Urease	Gelatin hydrolysis	Starch hydrolysis	Voges-Proskauer	Esculinase	Lecithinase	Solubility in 3 % KOH
1.	KY1	+	-	+	-	+	+	+
2.	KY2	-	-	+	-	+	+	+
3.	KY3	-	-	+	-	-	+	+
4.	KY4	-	-	+	-	+	+	+
5.	KY5	+	-	+	-	+	+	+
6.	KY6	+	-	+	-	+	+	+
7.	KK7	+	-	+	-	-	+	+
8.	KK8	+	-	+	-	-	+	+
9.	KK9	+	-	-	-	-	+	+
10.	EM10	-	-	+	-	-	+	+
11.	EM11	+	-	+	-	+	+	+
12.	HD1	+	-	+	-	+	+	+

+ Positive - Negative

were isolated. The abundance may be due to the presence of many insects. The increased potential for plasmid transfer among the bacterial strains in such habitats may explain this abundance.

Amplification was obtained for cry1 gene primer, at an annealing temperature of 55°C. Amplification of a single band of less than 250bp was obtained with universal cry1 gene primer for eight isolates KY2, KY3, KY5, KY6, KK7 KK8, KK9 and EM11 along with the reference strain HD1 (Fig. 3B and Table 6). cry1 gene is reported to be toxic to lepidopteran larvae. Salem *et al.* (2006) identified cry1 and cry4 gene by producing fragments of 277bp for cry1 and 439bp for cry4 genes. Similar reports had been made by earlier workers (Carrozi *et al.*, 1991, Chak *et al.*, 1994). No amplification was obtained for isolates KY1, KY4 and EM10 indicating that they may contain cry gene other than cry1 gene group. Screening of *Bacillus thuringiensis* isolates was carried out for cry4 gene with universal cry4 primer (Ben-Dov *et al.*, 1997). PCR amplification of standard strain 4Q1 with universal cry4 primer showed a single band of 524 bp. Amplification for cry4 gene was obtained for isolates KY1 and EM10. Single band of 575 bp and was obtained for isolate KY1. For isolate EM10, three bands of size 531, 942 and 1245 bp were obtained (Fig 3C). The remaining nine isolates could not amplify cry4 gene, which can be attributed to the presence of some other gene other than cry4 group.

In the present study, amplicons from PCR of cry1 and cry4 gene specific primers were obtained. These can be cloned into a suitable expression vector to understand the potency of genes and their further exploitation.

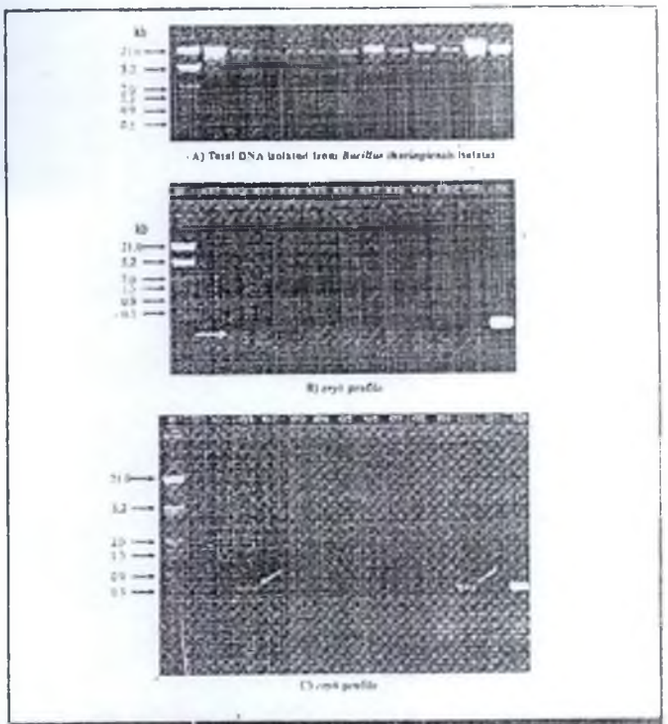

Fig 3 : Cry gene profile of native *B. thuringiensis* isolates

Emphasis must be laid on the identification and isolation of novel cry genes from bacterial strains which will be useful in pyramiding of different resistant genes. These can be utilized for developing transgenic crops, with increased resistance to insect attack.

Abbreviations:

LBA, Luria-Bertani Agar; T3 medium: Tryptone, Tryptose and Yeast extract medium; M: molar; ml:milliliter,

Table 6 : Details of amplicons obtained in *cry1* and *cry4* gene profile

Sr. No.	Isolate	Details of amplicons					
		cry1			*cry4*		
		No. of bands observed	Mol. wt of amplicon (bp)	Expected size of amplicon (bp)	No. of bands observed	Mol.wt of amplicon (bp)	Expected size of amplicon (bp)
1.	KY1	-	-	-	1	575	439
2.	KY2	1	~250	277	-	-	-
3.	KY3	1	~250	277	-	-	-
4.	KY4	-	-	-	-	-	-
5.	KY5	1	~250	277	-	-	-
6.	KY6	1	~250	277	-	-	-
7.	KK7	1	~250	277	-	-	-
8.	KK8	1	~250	277	-	-	-
9.	KK9	1	~250	277	-	-	-
10.	EM10	-	-	-	3	531, 942, 1245	439
11.	EM11	1	~250	277	-	-	-
12.	HD1	1	~300	277	-	-	-
13.	4Q1	-	-	-	1	524	439

; basepair, PCR; Polymerase Chain Reaction, dNTPs; deoxynucleoside triphosphate.

Acknowledgment:

The funding provided by the Department of Biotechnology, Government of India is kindly acknowledged.

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