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Genotyping and Molecular Characterization of Local *Bacillus Thuringiensis* Isolates of DOR by using PCR-Based Methods

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Abstract

Eight *Bacillus thuringiensis* (Bt) strains were isolated from the soil samples collected from different fields in Andhra Pradesh and Karnataka, India. The isolates were characterized on the basis of primary bioassay against 2nd instars larvae of *Helicoverpa armigera* and *Spodoptera litura* and also screened with REP-PCR (Repetitive Extragenic Palindromic sequence) to distinguish closely related relationship among the strains. Based on the bioassay of eight isolates, Bt-52 and Bt-127 isolates were showed high toxicity against 2nd instars of *H. armigera* and *S. litura*. Rep-PCR profiles with these isolates showed Bt-52 and Bt-127 isolates formed separate clusters in phylogenetic tree. Further PCR amplification was carried out with *cry* gene specific primers used for 8 Bt isolates revealed the presence of amplified fragments characteristic of ten different cry genes. Results showed the expected sizes of PCR products of cry1Aa, cry1Ac, cry1Ad, cry1Ae, cry2Aa, cry2Ab, cry2Ac, cry1C, cry1D and cry1E genes ranging from 1000-1286, 844, 500, 443 and 300 bp, respectively. Out of the eleven cry genes investigated, only cry1Ab gene was not found any of the 8 Bt isolates. One Bt isolate Bt-112 showed only cry2Aa gene with PCR product size of 500bp. While, six of the 8 isolates were harboring a combination of different cry genes, which are Lepidoptera active cry1 genes and Lepidoptera-Diptera active Lepidoptera active cry2 genes. Consequently, combined strains are good candidates in the search for bio-control agents with a wider spectrum of action against many different insects. On the other hand, cry1 genes were the most frequently found among the 8 local Bt isolates and the most common profile of cry2 genes contained cry2Aa and the second abundant genes are cry1Ad, cry2Ab, cry2Ac and cry1C.

Keywords: Bioassay, Insect Pests, PCR, Phylogenetic Tree, Primers, Rep - PCR

Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive, aerobic bacterium that has been used as a successful biological insecticide for more than 100 years and is a uniquely specific, safe and effective tool for the control of a wide variety of insect pests in the order of Lepidoptera, Diptera, Coleoptera and Hymenoptera as well as other types of pests including nematodes and mites (Ruiz *et al*. 2006; Ali *et al*. 2010; He *et al*. 2011; Prathap *et al*. 2011). The insecticidal property of *B. thuringiensis* is due to the production of crystal (Cry) proteins or δ- endotoxins that are encoded by cry genes, during sporulation (Schnepf *et al*. 1998). Many of the crystal proteins are predominantly synthesized in the form of crystalline protoxins (Bravo *et al*. 2011) that are solubilized in the alkaline midgut of susceptible larvae and cleaved by trypsin like gut proteases to produce a mature toxin that specifically binds to a receptor on the membrane leading to pore formation, cell lyses and eventually insect death (Schnepf *et al*. 1998)

Since the first *cry* gene was cloned from *B. thuringiensis ssp. kurstaki* HD-1 in 1981 (Schnepf &Whiteley, 1981) the search for new *cry* genes is an ongoing effort worldwide with 570 *cry* genes discovered so far (Crickmore *et al*. 2011) and the bacterium could carry different *cry* genes leading to production of different Cry proteins. *cry* genes have been classified based on sequence similarities and their target insect groups. The discovery of *cry* genes has also led to the rapid development of transgenic crops with insecticidal traits against insects (Schnepf *et al*. 1998; Bravo *et al*. 2007). Since transgenic plants expressing insecticidal proteins were first commercialized in 1996, transgenic maize, cotton and potato carrying *cry* genes have been planted on more than 32 million hectares in 2006 to 134 million hectares in 2010 (James, 2010). The use of *B. thuringiensis* bioinsecticides has led to development of insect resistance to individual toxins (Zhao *et al*. 2000). The discovery of novel *cry* genes with new or broad activity spectra or higher toxicity is important for the development of new products and the management of insect resistance (Xue *et al*. 2008; Darsi *et al*. 2010).

In the last few years, several PCR based techniques, mainly Rep –PCR analyses, multiplex PCR, which allowed the accurate determination of families of *cry* genes (Krisana *et al*, 1999) or specific endotoxin genes have been proposed (Bourque *et al*. 1993; Ceron *et al*. 1994; Goncalves *et al*. 2009; Abousaid *et al*. 2011).
Most of the commercial Bt formulations used for the control of Lepidopteron pests predominantly contain toxins of Cry1A and Cry2 family, especially Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ac proteins. The efficacy and host range of the strain is dependent on the crystal protein genes the strain carries (Chang *et al*. 1998; Schnepf *et al*. 1998; Aly 2007). Usually the Bt strains carry a combination of *cry* genes and therefore become effective against different insect pests. Therefore, identifying the *cry* genes carried by a strain provides a clue regarding the utility of the strain against different groups of insects.

Materials and Methods

Bacterial Strains

Eight *Bacillus thuringiensis* (Bt) isolates were isolated from soil samples from selected regions of Andhra Pradesh and Karnataka districts, India (**Table 1**). The strains were maintained at Directorate of Oilseeds Research (DOR). In the present study, Bt-3, Bt-34, Bt-41, Bt-52, Bt-55, Bt-112, Bt-116 and Bt-127 were used for insect bioassay with *H. armigera* and *S. litura* and the Repetitive Extragenic Palindromic sequence (Rep-PCR) analysis. Based on the results the Bt strains were further charecterized by PCR to identify the *cry* genes harboring in the strains.

Table 1 : Distribution of *B.thuringiensis* soil isolates from selected regions of Andhra Pradesh and Karnataka district. India.

S.No	Isolate Names	Village	District	Crop
1	Bt-3	Nandimullaguda,Wanaparthy	Mahaboonagar	Castor
2	Bt-34	Beijanki	karimnagar	Red gram
3	Bt-41	beijanki	karimnagar	Cotton
4	Bt-52	Amarguda	Darwad	Chilli
5	Bt-55	Amarguda	Darwad	chilli
6	Bt-112	Medikondur	Guntur	Chilli
7	Bt-116	Navakudur	Guntur	Red gram
8	Bt- 127	Metipalli	Karimnagar	Chilli & Cotton

Insect Bioassay

Bioassays were conducted with all the eight local isolates of Bt against neonate larvae of *H.armigera* and *S.litura.* The isolates were multiplied in nutrient broth at 30^0C and 300 rpm for 72 h, centrifuged for 10 minutes at 10,000 rpm. The resultant pellets were dried in a laminar airflow, powdered and used for the larval bioassays after suspending in PBS buffer. The test doses of Bt powders were 1.0 mg/ml for *H. armigera* and 1.5 mg/ml for *S. litura*.

Bioassays against *H. armigera*

Larvae of *H. armigera* were maintained in the laboratory on a semi-synthetic diet. The diet was poured as a thin layer into 12 well tissue culture plates, approximately at 4ml per well with a surface area of 3.14 sq.cm. Test suspensions of Bt (1 mg/ml) @100µl were overlaid on the diet surface in each well for all isolates. One larva was released in each well. A total of 30 larvae were used @10 larvae/replication.

Bioassays against *S. litura*

Fresh castor leaves were washed, shade dried and cut into leaf discs of 50.24 cm^2. The leaf surfaces were treated with 0.5 ml of the test suspension of Bt powders and allowed to shade dry. These discs were placed on moist cotton in Petri plates. Ten larvae of *S. litura* were released on each leaf disc. A total of 30 larvae were used @10 larvae/replication. For control, leaf discs were treated with PBS buffer.

Observations: The treated larvae were maintained at 27 ± 1^0C. Larval mortality was recorded at 24, 48, 72 and 96 hours after treatment.

Bacterial Genomic DNA Extraction

Genomic DNA was isolated by following the standard method (Ausubel *et al*. 1999). 5 ml of overnight grown bacterial culture was used for genomic DNA isolation. The culture was centrifuged in 1.5 ml eppendorf tube for 3 min at 10000 rpm. The pellet was resuspended in 567µl of $T_{10}E_1$ buffer by repeated pipetting. To this suspension 30µl of 10% SDS and 3µl of 20 mg/ml proteinase K were added, mixed well and incubated at 37°C for 1 hour followed by addition of 100µl of 5M NaCl and thoroughly mixed. To this, 80µl of CTAB/ Nacl solution was added, mixed well and incubated at 65°C for 10 min. Equal volume of Phenol/ Chloroform/ Isoamyl alcohol (P: C: I) was added, mixed well slowly until it became monophasic and centrifuged at 10000 rpm for 5 min. The aqueous phase was taken to a fresh eppendorf tube, equal volume of Chloroform/ Isoamylalcohol (C:I) was added, mixed well and centrifuged at 10000 rpm for 5 min. This step was repeated twice. Finally, the supernatant was taken into a fresh eppendorf tube and the DNA was precipitated with equal volumes of Ethanol. After keeping at 4°C for 30 min, it was centrifuged at 12000 rpm for 10 min to pellet the DNA. The supernatant was decanted. The pellet was washed with 70% alcohol twice, air-dried and dissolved in TE [10Mm Tris-Hcl, 1mM EDTA (pH 8.0)].

REP – PCR profiling of Bt isolates

REP - PCR was carried out independently with three sets of primers including REP (**F:** ICGICTTATCIGGCCTAC, **R:** IIIICGICGICATCIGGC), ERIC (**F:** AAGTAAGTGACTGGG GTGAGCG, **R:** TGTAAGCTCCTGGGGATTCAC) and BOX (CTACGGCAAGGCGACGCT GACG). Each PCR was repeated at least twice to confirm the results. Total genomic DNA isolated from Bt isolates was used as template for PCR amplification. 50 ng DNA was used as template in 20 ul of reaction mixture containing 1.0 uM of each primer, 0.2 mM of each of the four dNTPs, (1X) *Taq* DNA polymerase buffer, 5 U *Taq* DNA polymerase (Invitrogen). PCR cycling profile was 1 cycle at 94°C for 5 min, 35 cycles of 94°C for 1 min, 52°C (ERIC and BOX primers) / 38°C (REP – PCR primers) for 1 min and 72°C for 1 min followed by a final extension step at 72° C for 8 min. PCR products were separated using Agarose gel electrophoresis in 1.5% TAE buffer and stained with 0.2 mg/ml Ethidium bromide according to Sambrook *et al* (2001). PCR products were visualized under UV transilluminator and the sizes of the amplicons were estimated based on λ – *Hind III* DNA marker as size markers.

PCR Analysis for Specific *Cry* Genes of Bt Isolates

Once the isolates were characterized to identify the subspecies they belonged to, the next step was to identify the presence of specific *cry* genes using gene specific primers (**Table 3**). Each PCR was repeated at least twice to confirm the results. Total genomic DNA isolated from *Bt* isolates was used as template for PCR amplification. 20-50 ng DNA was used as template in 20 ul of reaction mixture containing 0.5 uM of each primer, 0.2 mM of each of the four dNTPs, (1X) *Taq* DNA polymerase buffer, 2.5 U *Taq* DNA polymerase (NEB, USA). PCR cycling profile was 1 cycle at 94°C for 4 min, 30 cycles of 94°C for 1 min, 54-60°C for 1 min and 72°C for 2 min followed by a final extension step at 72° C for 10 min. PCR products were separated using Agarose gel electrophoresis in 1% TAE buffer and stained with 0.2 mg/ml Ethidium bromide according to Sambrook *et al* (2001). PCR products were visualized under UV transilluminator and the sizes of the amplicons were estimated based on 100 bp ladder loaded as size markers.

Results and Discussion

Insect Bioassay

Larval bioassays with the eight local isolates of Bt revealed a high larval mortality of 90-100% by 3 days after treatment excepting for the Bt isolate 112 that resulted only in 27.7% larval mortality. The mortality against *S. litura* was however very low ranging 13.3-36.7 % by 3 days after treatment with majority of the isolates. The Bt isolates 52 and 127 were promising resulting in larval mortality above 80.0% (**Table 2**). Since these two isolates were promising against both *H. armigera* and *S. litura*, Cry gene profiling of these eight Bt isolates was undertaken

Table 2 Insecticidal assay results carried out with 2nd instar larvae of *Helicoverpa armigera* and *Spodoptera litura*.

Isolate Name[a]	% neonates mortality of *Helicoverpa armigera*	% neonates mortality of *Spodoptera litura*
Bt – 3	100	13.3 ± 5.3
Bt – 34	90.0 ± 8.5	20.0 ± 8.5
Bt – 41	100	36.7 ± 3.4
Bt – 52	100	86.7 ± 5.3
Bt – 55	100	13.33 ± 3.4
Bt – 112	27.7 ± 3.9	33.3 ± 7.9
Bt – 116	90.0 ± 7.4	60.0 ± 11.8
Bt – 127	100	83.3 ± 5.3

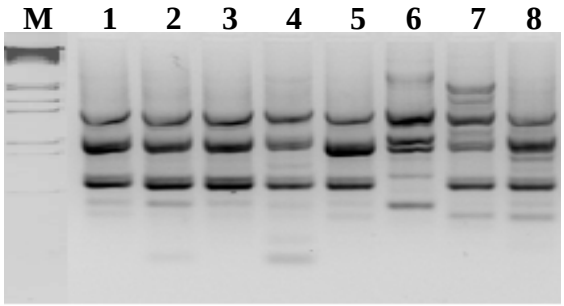
Table 3 Details of *cry* gene specific primers.

Primer pair [a]	Sequence of the primer	*cry* gene(s)	Position [b]	Product size (bp)
Bt-DOR1 (F)	TTCCCTTTATTTGGGAATGC	cry1Aa	1023-1043	1286 bp
Bt-DOR1 (R)	TTTGCATTGTAGCGAATTAA	cry1Aa	2289-2309	
Bt-DOR2 (F)	GGTTCTGTAATTTCAGGACC	cry1Ac	1452-1472	844 bp
Bt-DOR2(R)	TTTTGCATTGTAGCGAATTA	cry1Ac	2296-2316	
Bt-DOR3 (F)	TATGTTCAAG CTGCAAATTT	cry1Ad	482-502	1212 bp
Bt-DOR3 (R)	GCTTCCGGACTGTAAATTAC	cry1Ad	1673-1693	
Bt-DOR4 (F)	TATTTCCTTG TCTCTAACGC	cry1Ae	192-212	1100 bp
Bt-DOR4 (F)	CCCGTCAAGAACAGATAGAC	cry1Ae	1216-1236	
Bt-DOR5 (F)	GTTATTCTTAATGCAGATGAA	cry2Aa	571-592	498 bp
Bt-DOR5 (R)	GAGATTAGTCGCCCCTATGAG	cry2Aa	1048-1069	
Bt-DOR6 (F)	GTTATTATCAGGCTAATTTA	cry2Ab	807-827	445 bp
Bt-DOR6 (R)	TCTTCTACATTTGCTTGCAG	cry2Ab	1231-1251	
Bt-DOR7 (F)	TGTCATGCACATAATGTAGT	cry2Ac	37-57	300 bp
Bt-DOR7 (R)	GCTCTTAAAATCTCTTGCAT	cry2Ac	317-337	
Bt-DOR8 (F)	ATGGAGGAAAATAATCAAAA	cry1C	47-67	1100bp
Bt-DOR8 (R)	GGATTTGATAAAGTCCTAAA	cry1C	1127-1147	
Bt-DOR9 (F)	CTGCAGCAAGCTATCCAA	cry1D		1138 bp
Bt-DOR9 (R)	ATTTGAATTGTCAAGGCCTG	cry1D		
Bt-DOR10 (F)	GGAACCAAGACGAACTATTGC	cry1E		500bp
Bt-DOR10(R)	GGTTGAATGAACCCTACTCCC	cry1E		

[a] F and R – referred to forward and reverse primers, respectively.
[b] Primer position of the sequence of respective *cry* gene in the NCBI GenBank database.

Rep – PCR profiling of Bt isolates

All the PCR patterns obtained from the eight Bt strains from 2 to 11 bands ranging from 200bp to 3500 bp size bands (Fig.1). The Dendogram obtained are shown on (Fig.2). The overall topology of majority of Bt strains was similar in Dendogram expect Bt-52 and Bt-127 which shows separate sub-cluster in phylogenetic tree with Rep-PCR.



A

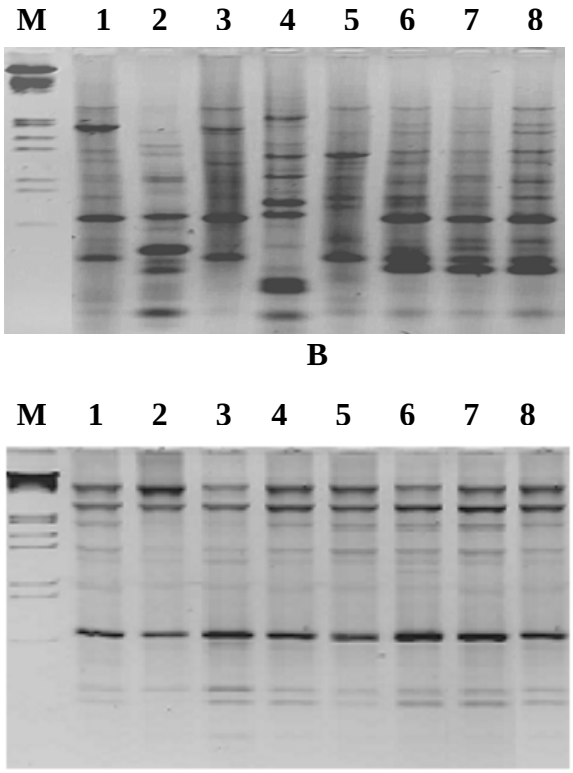


Fig. 1 REP-PCR profiling of *Bacillus thuringiensis* strains on 1.5 % Agarose gel electrophoresis. (**A**) PCR Amplification with REP primers. (**B**) PCR Amplification with ERIC primers. (**C**) PCR amplification with BOX primers. Lane-M λ- *Hind III* DNA marker, Lane-1 Bt-3, lane-2 Bt-34, lane -3 Bt-41, lane-4 Bt-52, lane-5 Bt-55, lane-6 Bt-112, lane-7 Bt-116 and lane-8 Bt-127.

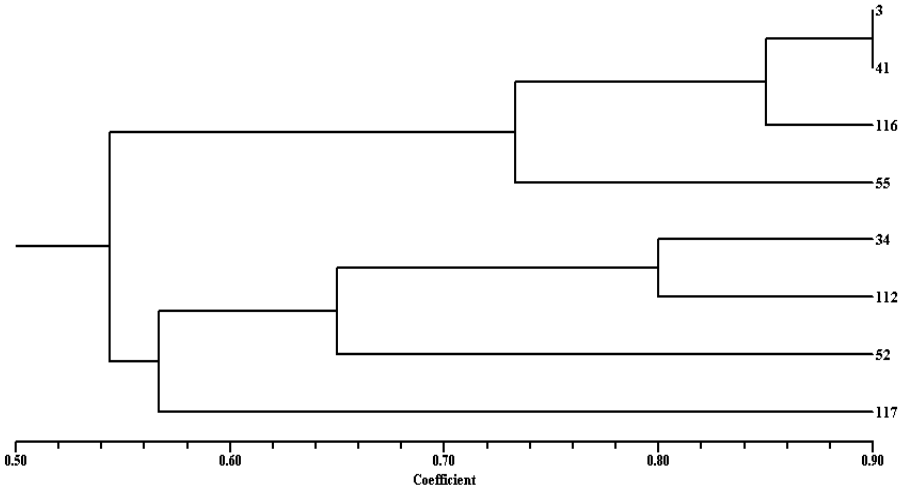


Fig. 2: Dendrogram showing UPGMA clustering of *Bacillus thuringiensis* isolates **using REP – PCR data (NTSYSpc2.2 software).** Two major clusters are indicated and the scale indicates percentage of similarities.

In NTSYS, it was deviated into two major clusters A and B. The first cluster A was again divided into a series of sub-clusters, which contained the strains of Bt-3, Bt-41, Bt-116 and Bt-55 whereas Bt-52 formed a separate sub-cluster with 0.64 similarity coefficient value. The second cluster B was also again divided into two sub-clusters (a) and (b), in sub-cluster (a) Bt-34 and Bt-112 shows 0.75 value in similarity coefficient scale. Whereas in sub-cluster (b) the strain Bt-127 showing coefficient value of 0.60 similarity among all other Bt strains. The Rep-PCR finger print method is to distinguish strains of *B.thuringiensis* has some advantages, it is simple, rapid and reproducible, and it could be used to differentiate numerical *B.thuringiensis* strains without raising antisera against flagella (Krishna *et al* 1999). The diversity studies using Rep-PCR is due to the complexity of banding patterns in microbial community DNA. However, whole genome amplification (eg.Multiple displacement amplification) can be coupled to the Rep-PCR technique to allow for the determination of the genotypes of single cells without cultivation (Rebecca *et al* 2011).

Identification of *cry* gene (s) in *Bacillus thuringiensis* local isolates.

The PCR amplification analysis of the 8 Bt isolates revealed the presence of amplified fragments characteristic of eleven different cry genes. Results showed the expected sizes of cry1Aa, cry1Ac, cry1Ad, cry1Ae, cry2Aa, cry2Ab, cry2Ac, cry1C, cry1D and cry1E genes ranging from 1000-1286, 844, 500, 443 and 300 bp, respectively (Table 4). Out of the eleven cry genes investigated, only cry1Ab gene was not found in any of the 8 isolates. The one Bt-112 isolate showed only cry2Aa gene with size of 500bp (Fig.3) which was active against Lepidoptera-Diptera insects. While seven of the 8 Bt isolates were harboring a combination of different cry genes. Cry1genes are active against Lepidoptera where as cry 2 genes are active against Lepidoptera-Diptera insects. In four different Bt isolates showed 7 different *cry* genes such as Bt-3, Bt-41, Bt-55, Bt-116 where as second abundant cry genes present in isolates of Bt-52 and Bt-127. The seven isolates Bt-3, Bt-34, Bt-41, Bt-52, Bt-55, Bt-116 and Bt-117 harboring the Lepidoptera and Diptera active gene could be used for wide spectrum of both insects. The cry 2, A Diptera an Lepidoptera specific protein and cry3, Coleoptera Specific protein were reported (Hofte and whitely, 1989 and Head 2005).

Table 4 The size of PCR products amplified by using *cry* gene specific primers from different Bt isolates.

		Cry genes with PCR product size (bp)										
		Lepidoptera-active genes							Lepidoptera-Diptera active genes			
S.No	Isolates	cry1Aa	cry1Ac	cry1Ad	cry1Ae	cry1C	cry1D	cry1E	*cry2Aa*	*cry2Ab*	*cry2Ac*	**
1	Bt-3	.		.		.	.		.	.	.	**70%**
2	Bt-34			.					.	.		30
3	Bt-41	.		.		.	.		.	.	.	**70**
4	Bt-52	.		.	.	.			.		.	**60**
5	Bt-55			.		.	.	.	.	.	.	**70**
6	Bt-112								.			10
7	Bt-116			.	.	.	.		.	.	.	**70**
8	Bt-127		.		.	.		.	.	.		60
	*	37%	13	75	38	75	50	25	100	75	63	

· The presence of each cry gene.
* Percentage of each cry gene in all isolates.
** Percentage of all cry genes in each isolate.

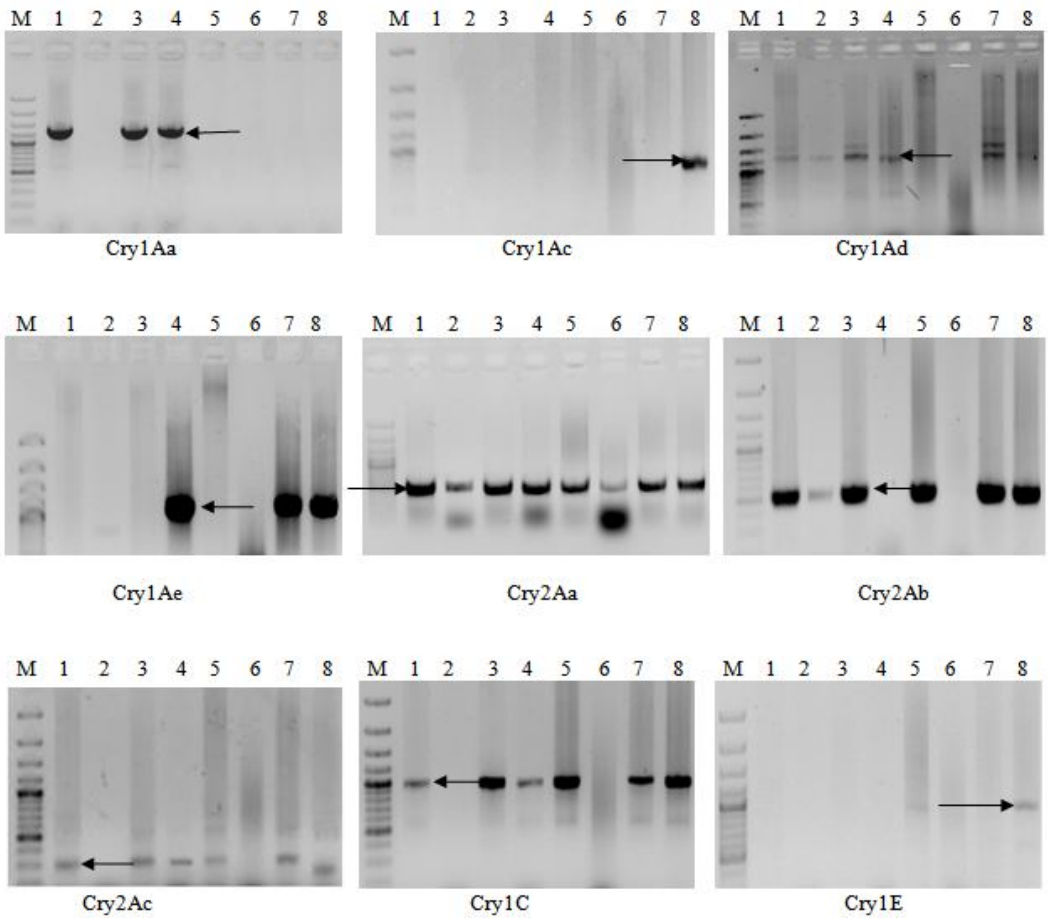


Fig. 3 PCR amplification of local Bt isolates with *cry* gene specific primers. Primers specific to *cry1Aa*, *cry1Ac*, *cry2Aa*, *cry2Ac*, *cry1C* and *cry1E* genes were used for amplification with the genomic DNA. Lane-M 100 bp.

The presence of different *cry* genes in the same *B.thuringiensis* strain, cry1, cry3, cry8 or cry7 genes has been reported (Nariman 2007). The presence of more than one *cry* type gene in individual isolates was not surprising since in addition to their own chromosomal *cry* gene, strains can carry plasmids that encode additional cry genes. (Carlton *et al*, 1985; Carlson *et al.*1993). Moreover isolates can harbor more than one type of plasmid (Benjawan *et al.* 2010). Nowadays, to control the Lepidoptera pest in agriculture Bt is effectively used as a critical biopesticide in biocontrol. It is significantly necessary to screen a Bt isolate with high toxicity and clone its novel insecticidal toxin genes to construct genetically engineered bacteria and transgenic plant (Liu xu-Guang *et al.* 2010).using Bt genes with resistance to four major stem borers were successfully incorporated into elite CIMMYT maize inbred line (CML216) and tested in insect bioassays in Kenya (Hugo De Groote *et al.* 2011).
Currently, the most commonly used and commercially exploited insect resistance genes are the *cry* genes from *B. thuringiensis* var. *kurstaki*. Insecticidal endotoxins of *B. thuringiensis* have acquired great significance because of their specificity to target pests, non-toxicity to humans and beneficial insects, toxicity at low concentrations and environment friendly nature. Cry toxins have not been found promising against *Spodoptera litura* larvae; hence there is a continued interest for identifying potent toxins. Prabagaran et al., 2002 reported efficacy of Cry1C and Cry2 toxins against S. *litura*. Cry1Aa was reported to cause 50% mortality of the larvae at a high concentration of 1500ng/cm^2 and increasing its concentration up to 3000ng/cm2 also failed to increase the larval mortality (Lakshminarayana and Sujatha, 2005). Larvae of *Helicoverpa armigera* are particularly susceptible to Cry1Ac (Brevault *et al*, 2009) while other proteins like Cry1Aa, Cry1Ab, and Cry2 have shown different degrees of toxicity (Clara *et al.* 2005)

In the present study, two isolates Bt-52 and Bt-127 resulted in complete mortality of *H. armigera* larvae coupled with a high mortality of *S. litura* larvae ranging 83-86%. The isolate Bt-52 harbors Cry1Aa and Cry1C genes while Bt-127 harbors Cry1Ac, Cry1C and Cry2 genes. Larval mortality is known to be a function of Cry gene expression and binding of toxin to midgut receptors (Craig *et al.* 2007).
Spodoptera litura and *Helicoverpa armigera* are economically important polyphagous pests. However the Cry toxins reported effective against these pests are different. Since these two pests occur on several crops, formulations can be developed with the promising isolatesBt-52 and Bt-127 for effective management of both the pests.

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