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Research Article

Characterisation of native *Bacillus thuringiensis* isolates toxicity to fall armyworm, *Spodoptera frugiperda* (J.E. Smith)

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ABSTRACT: Toxicity of nine indigenous *Bacillus thuringiensis* (*Bt*) isolates collected from Tamil Nadu, India were tested against fall armyworm, *Spodoptera frugiperda*. At 30 µg/ml concentration, two *Bt* isolates *viz.,* T350 and T532 recorded 100 per cent mortality whereas isolates T527 and T532 registered 96 per cent mortality against neonate larvae of *S. frugiperda* in leaf dip bioassay. SDS PAGE analysis of spore crystal mixture revealed the presence of Cry1 and Cry2 proteins with visible bands at 130 kDa and 65 kDa. PCR screening results showed the presence of *cry1* (*cry1A, cry1Aa, cry1Ab*), *cry2* (*cry2Aa, cry2Ab*) in four isolates and *vip3A* genes in three isolates but *cry9* gene was not present in any of the isolates tested.

KEY WORDS: *Bacillus thuringiensis*, bioassay, *Cry* Genes, fall armyworm, PCR, SDS PAGE

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INTRODUCTION

Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is an important lepidopteran insect pest in maize growing areas. This pest has its root of origin from the tropical and subtropical regions of America and it was first reported in India during May - June 2018 at Shivamogga, Karnataka, India (Sharanabasappa *et al*., 2018). In Tamil Nadu, its occurrence was first reported in November, 2018 at Erode and Karur districts (Srikanth *et al.,* 2018). It is a polyphagous insect pest known to attack nearly 353 host plant species (Montezano *et al*., 2018). First to third instar larval stages of FAW, consume less than 2 per cent of total foliage whereas fourth, fifth, sixth instar larval stages consume 4.7, 16.3 and 77.2 per cent of total foliage, respectively causing severe defoliation of the crop (Sparks, 1979). The yield loss caused by the *S. frugiperda* was accounted to be 49 per cent in maize (Houngbo *et al.,* 2020), accounting to a loss of US$ 2.48-6.19 billion (Prasanna *et al.,* 2018). Being a highly polyphagous pest, its control measures mainly rely on pesticide application which resulted in the development of resistance against permethrin, cypermethrin, carbaryl, thiodicarb, chlorpyrifos and dichlorvos (Yu, 1991). Further, harmful impact of pesticides on the environment, necessitates the development of products that are safe to the environment.

Bacillus thuringiensis Berliner (*Bt*) based bio-pesticides are one of the options which have been widely used as a successful biological insecticide over last six decades constituting 95 per cent of all commercial bio-pesticides due to its specificity and effectiveness in the management of agricultural pests (Schunemann *et al*., 2014). *Bt* have been isolated from various habitats *viz.,* soil, dead insects, grains, stored product dust, deciduous and coniferous leaves (Bernhard *et al.,* 1997; Chaufaux *et al.,* 1997; Ramalakshmi and Udayasuriyan, 2010). *Bt* is a spore-forming, gram-positive, facultative and aerobic soil bacterium that produce insecticidal crystal (Cry) proteins during sporulation (Hofte and Whiteley, 1989). Crystalline protein inclusions contain one or more δendotoxins or Cry toxins, encoded by *cry* genes, responsible for pores formation in insect gut after ingestion. Formation of pores leads to osmotic imbalance resulting in septicemia, toxicemia and causing death (Knowles and Dow, 1993). *Bt* is distributed globally and toxins are produced by each isolates that are specific to a particular group of insect

pests without affecting higher organisms and the environment (Schnepf *et al*., 1998). The toxins produced by wide variety of *Bt* isolates are used in the production of bio-insecticides and genes responsible for those toxins are being used in the development of genetically transformed insect resistant *Bt* plants (Romeis *et al*., 2006). As they are highly specific and safer to the environment, Crystal proteins are considered as a successful alternative for chemical insecticide in managing the insect pests (Roh *et al.,* 2007). Hence, the present study was conducted to screen the native *Bt* isolates effective against the FAW.

MATERIALS AND METHODS

Maintenance of insect culture

Initial culture of *S. frugiperda* was obtained from FAW laboratory, Dept. of Agricultural Entomology and reared with CIMMYT diet (Tefera *et al.,* 2011) at *Bt* laboratory, Dept. of Plant Biotechnology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. After maintaining for six generations on CIMMYT diet under laboratory conditions (25±1 δ 75±5 % RH; 16:8 light: dark hours) uniform insect population was used for bioassay studies.

Bt isolates

Nine indigenous isolates of *B. thuringiensis viz.,* T44, T73, T161, T210, T350, T352, T527, T532 and RM6 along with a standard strain (HD1), positive check (T405) and negative check (4Q7) were received from the *Bt* Laboratory, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, India. The cultures were revived and maintained in T3 medium (one litre: 3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 6.9 g sodium dihydrogen phosphate, 8.9 g disodium hydrogen phosphate, 100 µl of 0.05 g of manganese chloride dissolved in 1 ml of water, 20 g of Agar, pH – 6.8-7.0).

Spore crystal mixture preparation and SDS-PAGE analysis

The spore crystal mixtures were harvested according to the protocol used by Ramalakshmi and Udayasuriyan (2010). Single colony of each *Bt* isolate was inoculated as mother culture into a 5 ml of T3 broth and incubated at 30δovernight with shaking at 200 rpm. After 12-14 hours of incubation, 1% of mother culture was transferred into conical flasks containing 25 ml of T3 broth and again incubated at 30δ 200 rpm for 48 hours. A drop of grown culture was taken and smeared on a clean glass slide, heat fixed, stained with Coomassie brilliant blue (0.133 %) and observed for the presence of crystals and spores under bright field microscope at 100X magnification (Euromex iScope).

The 48 hours grown cultures (with >90 % lysed culture) were centrifuged at 10000 rpm for 10 min at 4δand the pellets were again suspended in 25 ml of ice-cold Tris–EDTA buffer and washed thrice with the same buffer which contains 100 mM PMSF (Phenyl Methyl Sulphonyl Fluoride) and washed once with 0.5 mM solution of NaCl. Then, Spore-crystal pellets were suspended in 500 µl of sterile distilled water containing 1 mM PMSF and stored at -20°C (Ramalakshmi and Udayasuriyan, 2010).

SDS-PAGE was performed to characterize the Cry proteins in 10 % SDS-PAGE gels by following the standard method of Laemmli (1970). The gel was stained with 0.4% Coomassie brilliant blue R250. The molecular weight of proteins was determined using protein markers (Genetix Biotech Asia Pvt. Ltd.).

PCR screening for *cry* and *vip* genes

The bacterial genomic DNA from indigenous *Bt* isolates was isolated by following standard protocol (Kalman *et al.,* 1993). PCR was performed in master cycler (Eppendorf nexus Gx2) with a 20 µl reaction mixture containing 1µL of template DNA, 10 µL of PCR Master Mix (Smart prime 2x), 10 pmol of each primer (1 µL) and 7 µl of sterile distilled water. Lepidopteran toxic Cry protein encoding genes (*cry1, cry2, cry9* and *vip3A*) were screened using gene family primers (Table 1). Using specific primers, *cry*2 gene positives were again screened for *cry*2Aa and *cry*2Ab. After confirming the presence of *cry*1A gene with an amplicons size of 2.1 Kb, RFLP analysis of PCR product was done with *Eco*RI enzyme to further confirm the presence of *cry1Aa*, *cry1Ab*, *cry1Ac* genes, based on the presence or absence of 378 bp, 415 bp, 580 bp, 726 bp and 958 bp fragments (Table 2).

Bioassay

The concentration of protein present in spore-crystal mixture was assessed by Bradford method (Bradford, 1976) with Bovine Serum Albumin as standard (BSA) using ELISA reader (Biotek-Powerwave XS). The *in-vitro* insect bioassay against FAW was performed by leaf dip bioassay. Uniform stage young maize leaves were cut into ~2 × 2 cm size, and 20 µl (30 µg/ml) of crude protein was coated on both side of leaf surface and allowed to air dry. The treated leaf disc was placed on a moist filter paper to maintain turgidity, in a plastic container (3 cm diameter). Three days old egg masses ready to hatch were placed in a container, on the previous night, and neonates larvae (12 hrs) hatched from these egg masses were used on the next day morning for bioassay. Ten larvae were released on each leaf disc without any physical damage to the larvae using camel hair brush. The crude protein of standard strain HD1 and positive check T405 was used as a positive controls and that of 4Q7 as negative

Table 1. List of primer sequences used in the study

Gene	Primer sequence	Product size	Reference
cry1	FP: 5′-CATGATTCATGCGGCAGATAAAC-3′	277 bp	Ben-dov *et al*. (1997)
	RP: 5′-TTGTGACACTTCTGCTTCCCATT-3′		
cry1A	FP: 5′-GCCCCGGGCCTGGGTCAAAAATTGATATTTAG -3′	2.1 Kb	Ramalakshmi *et al.* (2014)
	RP: 5′-CGGGTCGACTAAATTGGATACTTGATCA -3′		
cry1Ab	FP: 5′-CCCCGGGCCTGGGTCAAAAATTGATATTTAG-3′	2.1 Kb	Darsi *et al.* (2010)
	RP: 5′-GCTGCAGTGCTCTTTCTAAATCATATCTGCC-3′		
cry2	FP: 5′-GTTATTCTTAATGCAGATGAATGGG-3′	700 bp	Ben-dov *et al.* (1997)
	RP: 5′-CGGATAAAATAATCTGGGAAATAGT-3′		
cry2A	FP: 5′-ATGGTACCATGAATAATGTATTGAATAGTGGA-3′	1.9 Kb	Manikandan *et al*. (2015)
	RP: 5′-GTTCTAGACTCAAACCTTAATAAAGTGGTG-3′		
cry2Aa	FP: 5′-GTTATTCTTAATGCAGATGAATGGG -3′	498 bp	Ben-dov *et al.* (1997)
	RP: 5′-GAGATTAGTCGCCCCTATGAG-3′		
cry2Ab	FP: 5′-GTTATTCTTAATGCAGATGAATGGG-3′	546 bp	Ben-dov *et al.* (1997)
	RP: 5′-TGGCGTTAACAATGGGGGGAGAAAT-3′		
cry9	FP: 5′-CGGTGTTACTATTAGCGAGGGCGG-3′	345 bp	Ben-dov *et al*. (1997)
	RP: 5′-GTTGAGCCGCTTCACAGCAATCC-3′		
vip3A	FP: 5′-CCTCTATGTTGAGTGATGTA-3′	1.0 Kb	Jain *et al.* (2012)
	RP: 5′-CTATACTCCGCTTCACTTGA-3′		

Table 2. RFLP of ~2.1 kb amplicon of *cry1A* gene with *Eco* RI enzyme (Ramalakshmi *et al.,* 2014)

S.No.	*cry1A* genes	RFLP fragment sizes (bp)
1	*cry1Aa1*	378, 415, 580, 726
2	*cry1Ab1*	378, 415, 583, 726
3	*cry1Ac1*	415, 726, 958

control, and absolute control was maintained with water. The larval mortality was observed at 72 hrs after treatment and expressed in percentage.

RESULTS

Bioassay

Leaf disc bioassay result revealed that, three *Bt* isolates *viz.,* T350, T352 and T405 produced 100 per cent larval mortality at 72 hrs after treatment, whereas T527 and T532 recorded 96 per cent larval mortality (Table 3). Further, feeding damage by the surviving larvae was less with minimal larval development in leaf disc treated with *Bt* protein when compared to negative controls (Fig. 1).

Figures in parenthesis are arc sine transformed mean values. In a column, mean followed by common letters are not significantly different by DMRT (0.05).

Crystal morphology

Bipyramidal shape of crystals were observed in the *Bt* isolates *viz.,* T350, T352, T405, T527, T532 and positive strain HD1 (Fig. 2).

SDS-PAGE analysis

SDS-PAGE analysis showed two prominent bands of ~130 kDa and ~ 65 kDa size in *Bt* isolates *viz.,* T350, T352, T405, T527 and T532, representing Cry1 and Cry2 proteins, respectively. Whereas, *Bt* isolate T210 which was not toxic to FAW showed five bands at different sizes ~150, 110, 25, 20, 17 kDa and rest of the isolates did not produce any visible bands (Fig. 3). Protein profiling of various *Bt* isolates help in studying the diversity of insecticidal Cry proteins and discovery of novel *cry* genes. The lepidopteran specific Cry proteins are found belonging to proteins Cry1 and Cry2 of ~ 130 kDa and ~ 65 kDa size, respectively.

Table 3. Mortality of fall armyworm, *Spodoptera frugiperda* caused by indigenous *Bt* isolates

S. No.	*Bt* isolates	Mean larval mortality (%) at 30 µg/ml of crude protein
1	HD1 (Standard strain)	100 (90.00)[a]
2	T405 (Positive control)	100 (90.00)[a]
3	T44	0.0 (0.52)[d]
4	T73	0.0 (0.52)[d]
5	T161	3.33 (10.52)[d]
6	T210	23.33 (28.88)[c]
7	T350	100 (90.00)[a]
8	T352	100 (90.00)[a]
9	T527	96.66 (79.47)[a]
10	T532	96.66 (79.47)[a]
11	RM6	43.33 (41.16)[b]
12	4Q7 (Negative control)	0.0 (0.52)[d]
13	Water control	0.0 (0.52)[d]
	SEd	2.9235
	CD (0.05)	6.0094
	CV%	6.95

Figures in parenthesis are arcsine transformed mean values. In a column, mean followed by common letters are not significantly different by DMRT (0.05).

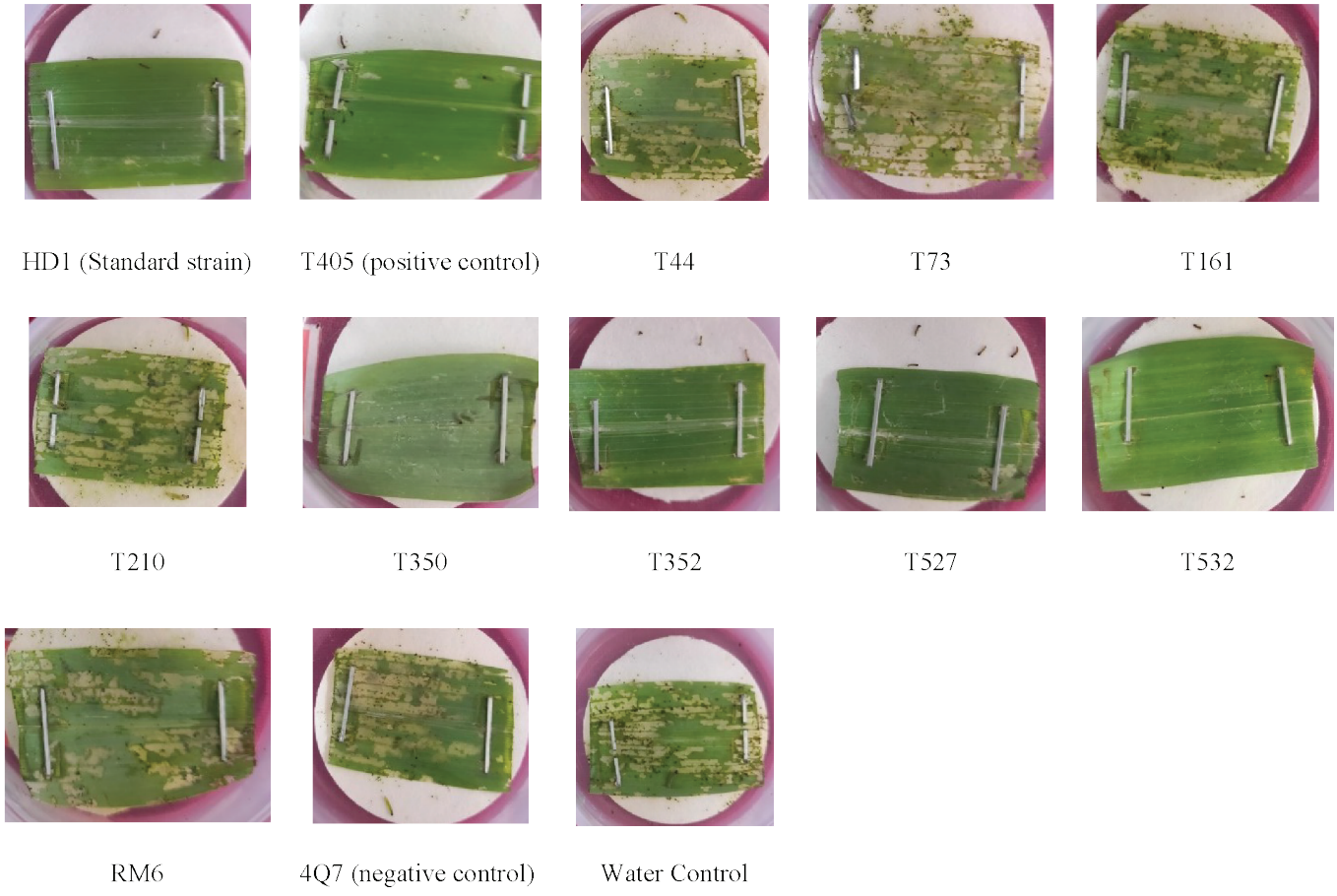
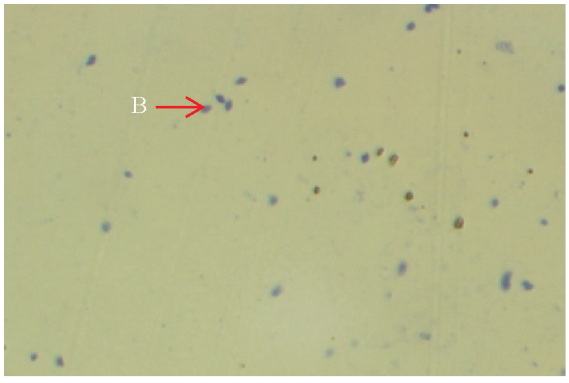
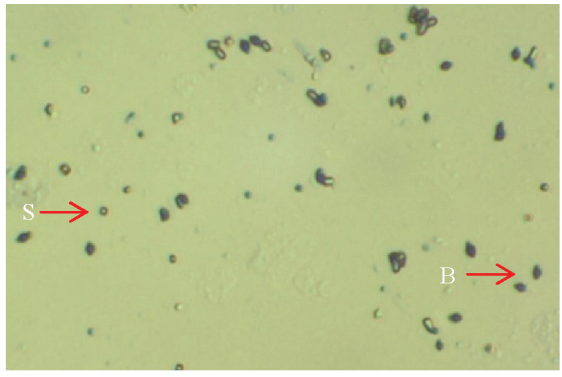


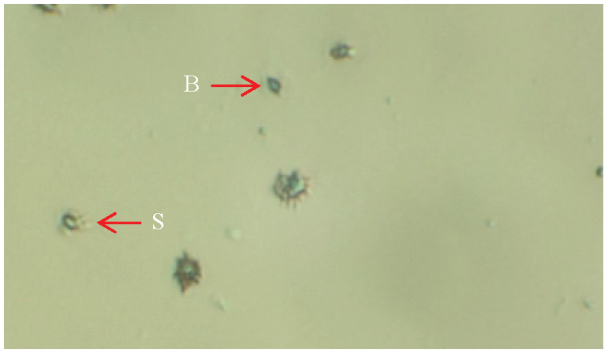
Fig. 1. Maize leaf damage in leaf dip bioassay (@ 20 µl of 30 µg/ml spore crystal mixture per leaf disc)



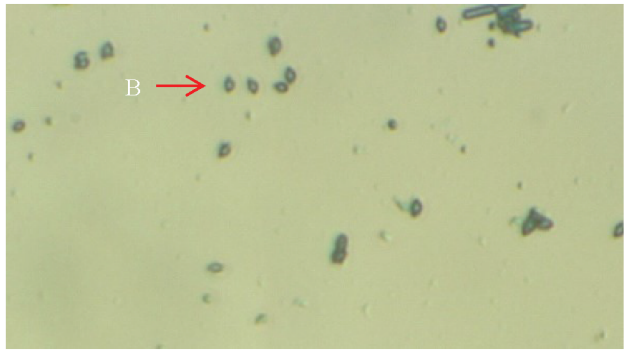
a. HD1



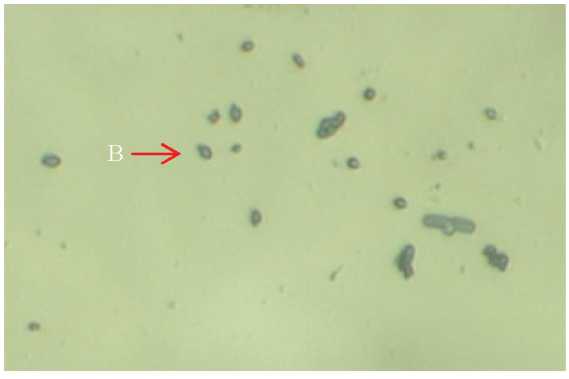
b. T405



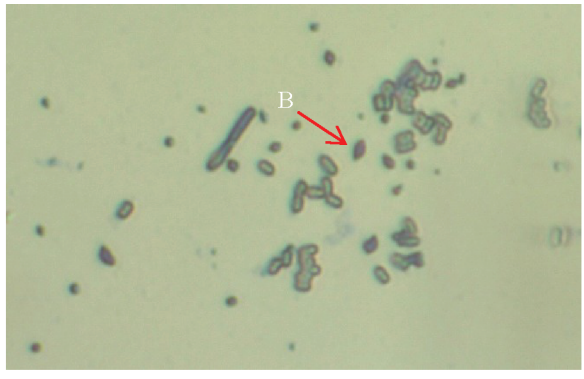
c. T350



d. T352



e. T527



f. T532

B- Bypyramidal shape of crystal ; S- Spore

Fig. 2. Crystal structure of effective Bt isolates (100x magnification)

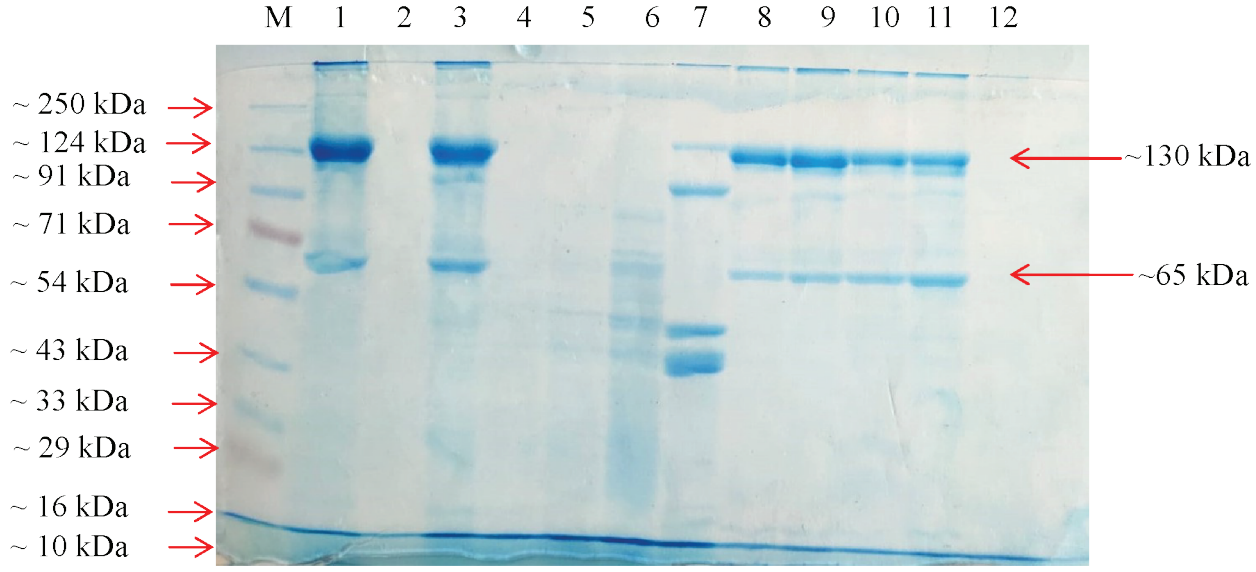


Fig. 3. Protein profiling of *Bt* isolates by SDS-PAGE. Lane M - high molecular range marker, Lane 1 to 12 - HD1, 4Q7, T405, T44, T73, T161, T210, T350, T352, T527, T532 and RM6

PCR screening for cry genes

PCR Screening was carried out with lepidopteran toxic Cry protein encoding cry genes (cry1Aa, cry1Ab, cry1Ac, cry2A, cry2Aa, cry2Ab and cry9) and vip genes (vip3A) (Table 4) (Fig. 4). Five Bt isolates viz., T350, T352, T405, T527 and T532 were positive with cry1 family primers with an expected amplicon of 277 bp size. The presence of cry1A gene was confirmed with an amplicon size of 2.1 kb and RFLP analysis of PCR product with EcoRI enzyme confirmed the presence of cry1Aa, cry1Ab and cry1Ac genes, which resulted in 378 bp, 415 bp, 580 bp and 726 bp fragments or 415 bp, 726 bp and 958 bp fragments. Bt isolates T350, T352, T405, T527 and T532 (Fig. 5) showed fragments corresponding to cry1Aa or cry1Ab along with cry1Ac genes similar to the reference strain HD1. It was further confirmed as cry1Ab as these isolates were PCR positive with cry1Ab specific primer. The Bt isolates T350, T352, T405, T527 and T532 were PCR positive with cry2 gene specific primers with an expected size of 700 bp, whereas the Bt isolates T350, T405, T527, T532 were PCR positive for vip3A gene. None of the isolates were PCR positive for cry9 gene.

DISCUSSION

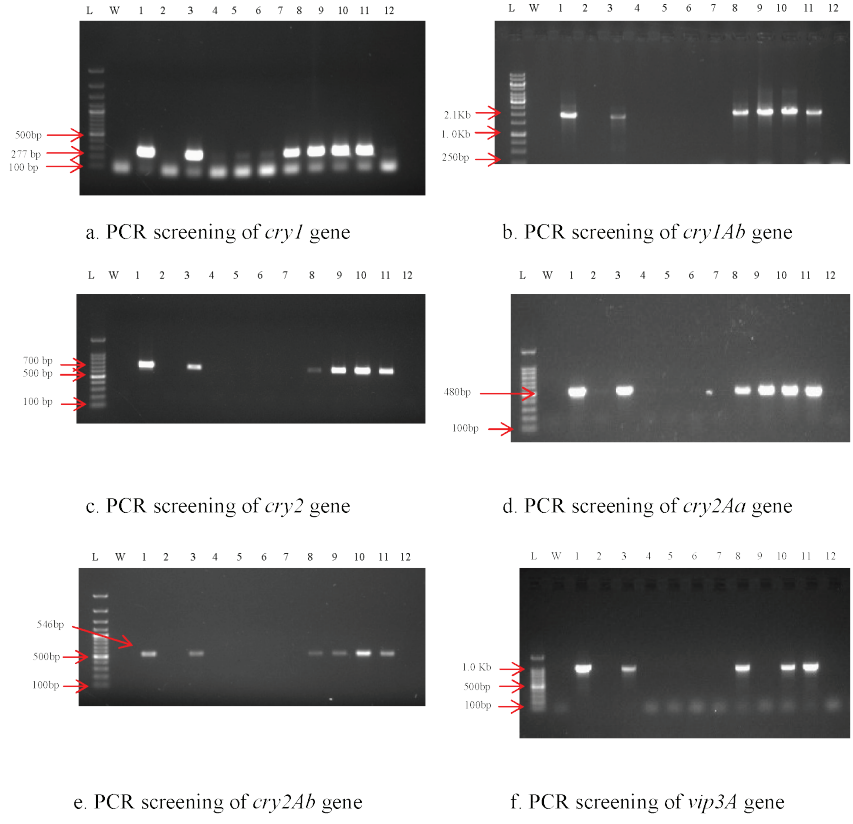
While screening local isolates of Bt, Manikandan *et al.* (2015) observed bipyramidal shape of crystals in T30 and T48 which was reported toxic against *Spodoptera litura,* in the present study, Bt isolates T350, T352, T527, T532 toxic against *S. frugiperda* were found to have bipyramidal shape of crystal inclusions. Bipyramidal shape of crystal structure was found to be dominant and more effective against lepidopteran larvae (Bernard *et al.,* 1997; Swamy *et al.,* 2013). Protein profiling of various Bt isolates help in studying the diversity of insecticidal Cry proteins and discovery of novel cry genes. The lepidopteran specific Cry proteins are found belonging to proteins Cry1 and Cry2 of ~130 kDa and ~ 65 kDa size, respectively. SDS-PAGE confirmed the presence Cry1 and Cry2 in the Bt isolates T350, T352, T527 and T532 showing visible bands at ~ 135 kDa and ~ 65 kDa similar to the reference strain HD1.

The results elucidated by Kaviyapriya *et al.* (2019) revealed that the indigenous Bt isolate T29 showed bipyramidal and cuboidal crystal inclusions and bands at ~ 130 kDa and ~ 65 kDa and showed 100 per cent mortality against first instar larvae of *S. frugiperda* by using leaf dip coating method and also reported that cry1, cry2Aa and vip3A genes in T29 isolate which may be a reason for the complete mortality. Similarly in the present study, Bt isolates T350 and T352 showed 100 per cent mortality and isolates T527 and T532 showed 96 per cent mortality against the neonates of *S. frugiperda* and contained cry1 and cry2 genes in all four toxic isolates but vip3A was reported in T350, T527 and T532 only. Results observed by Polanczyk et al. (2000) showed that Bt strains 4412 with cryIB and HD 68 with cryIA(a), cryID had toxic effect causing 80.40 per cent and 100 per cent mortality against S. frugiperda. Ramalakshmi and Udayasuriyan (2010) reported 17 isolates out of 70 Bt isolates tested were found to have the insecticidal proteins of ~135 kDa and ~65 kDa. Reyaz et al. (2017) found that five isolates of Bt viz., SWK1, KS2-3, 2M-6, KS2-6 and QZ-19 from Kashmir valley were found toxic against *S. litura* and *Helicoverpa armigera* with containing cry1, cry2Aa and cry2Ab genes and producing ~ 135 kDa and ~ 65 kDa proteins.

Table 4. Gene profiling of indigenous Bt isolates

Isolate/ strain	cry 1			cry 2			
	cry 1Aa	***cry 1Ab***	***cry 1Ac***	***cry 2Aa***	***cry 2Ab***	***cry 9***	***vip 3A1***
HD1	+	+	+	+	+	+*	+
T405	+	+	+	+	+	-	+
4Q7	-	-	-	-	-	-	-
T44	-	-	-	-	-	-	-
T73	-	-	-	-	-	-	-
T161	-	-	-	-	-	-	-
T210	-	-	-	-	-	-	-
T350	+	+	+	+	+	-	+
T352	+	+	+	+	+	-	-
T527	+	+	+	+	+	-	+
T532	+	+	+	+	+	-	+
RM6	-	-	-	-	-	-	-

+ = Present, - = Absent
*For cry9 screening 4ATI was used as positive control instead of HD1.



a. PCR screening of *cry1* gene

b. PCR screening of *cry1Ab* gene

c. PCR screening of *cry2* gene

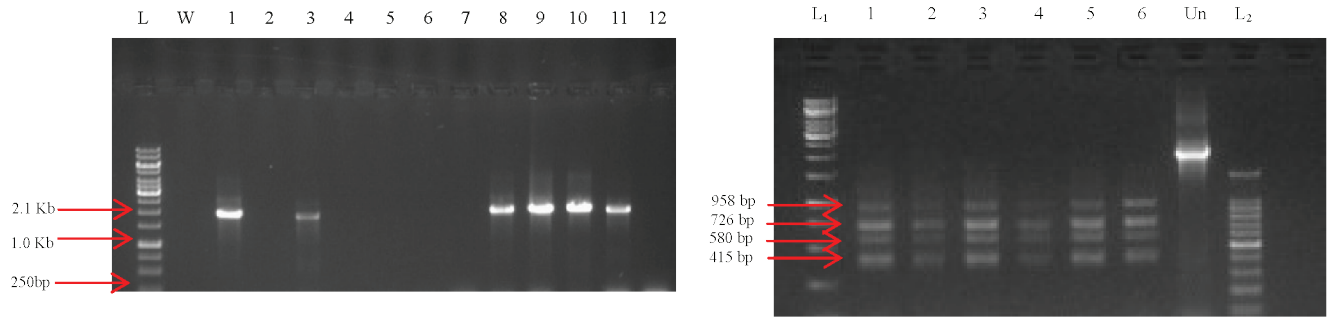
d. PCR screening of *cry2Aa* gene

e. PCR screening of *cry2Ab* gene

f. PCR screening of *vip3A* gene

Lane L: 100bp & 1kb ladders, Lane W: Water control, Lane 1: Positive control (HD1 for all genes), Lane 2: Negative control (4Q7), Lane 3 to 12 - T405, T44, T73, T161, T210, T350, T352, T527, T532 and RM6.

Fig. 4. PCR screening for *cry1*, *cry2* and *vip3* genes



a. PCR screening with cry 1A primer
Lane L: 1kb ladders,Lane 1 to 12 - HD1, 4Q7, T405, T44, T73, T161, T210, T350, T352, T527, T532 and RM6.

b. RFLP screening of cry 1A amplicons with EcoRI
Lane L1 & L2: 1kb ladders & 100bp, Lane 1mto 6 - HD1, T405, T350, T352, T527 and T532.
Un: Undigested PCR product

Fig. 5. Screening for *cry1A* family genes

The four indigenous Bt isolates T15, T16, T20 and T31 were identified to have cry1 gene and causing 90 to 100 per cent mortality against *H. armigera* (Ramalakshmi et al., 2014). Reyaz et al. (2019) reported that a Bt isolate T414 have bipyramidal and cuboidal crystal proteins indicating the presence of Cry1 and Cry2 proteins which showed 100 per cent mortality against *Pectinophora gossypiella*. Hassan *et al.* (2011) screened 25 isolates from Syrian soil against *Ephestia kuehniella* Zeller, *Phthorimaea operculella* Zeller, and *Cydia pomonella* L., out of which only five isolates (SSy125-c, SSy60-b, SSy141-c, SSy126-c, and SSy111-c) were found to be more toxic than the reference strain Bt kurstaki HD-1.

CONCLUSION

Based on the results obtained from this study, the *Bt* solates T350, T352, T527 and T532 can be explored for further development of formulation and transgenic crops.

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