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Characterization and Evaluation of Indigenous *Bacillus thuringiensis* Isolate T352 against Fall Armyworm, *Spodoptera frugiperda* (J.E. Smith)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Bacillus thuringiensis (Bt) Berliner is a ubiquitous soil bacterium with commercial bio pesticidal value and widely used for effective control of various important agricultural insect pests. Invasion of Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) into India caused potential yield loss in maize production, and also it threats the cultivation of other related crops. This study was aimed to characterize and evaluate the pathogenic activity of indigenous *Bt* isolate T352 against fall armyworm (FAW), *S. frugiperda.* The *Bt* isolate T352 was creamy white in colour and had irregular shaped flat colonies with undulated margin. Bipyramidal shape of parasporal inclusions was found in isolate T352. The isolate produced protein bands of ~ 130 kDa and ~ 65 kDa size in SDS PAGE analysis. PCR screening also confirmed the presence of *cry1Ab, cry1Ac, cry2Aa, cry2Ab* and *vip3A* genes. During probit analysis, isolate T352 exhibited the LC_{50} of 1.927 µg/ml as against 0.421µg/ml in positive standard strain HD- 1 based on mortality observed at 72 h after treatment in leaf disc bioassay with spore crystal mixtures.

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Keywords: Bacillus thuringiensis; Indigenous isolate; T352; fall armyworm.

1. INTRODUCTION

Maize is a multi-purpose staple food crop, widely cultivated throughout the world and has the highest productivity of 5.8 tonnes/ ha among all the cereals [1]. "However, numerous pest and disease outbreaks limit the yield and quality of maize. Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) a recent alien insect pest possess greater threat to the production of maize in Asia, particularly in India, is under risk" [2,3]. The rapid spread, migratory nature and gregarious feeding of the fall armyworm has resulted in significant crop losses around the world [4]. FAW is a polyphagous multivoltine insect pest that feeds about 353 different plant species belonging to 76 families, but it attacks maize, rice, sugarcane and forage grasses to a large extent [5,6]. The majority of chemical insecticides now in use failed to manage this pest, and these pesticides also lead to some environmental implications [7]. So biological control is the inevitable option to manage the population of FAW in an eco-friendly way.

"The most studied and promising microbial biopesticide worldwide is *Bacillus thuringiensis* (*Bt*) Berliner. *Bt* is an aerobic, gram positive, spore forming, soil dwelling bacteria" [8-11] that produces "virulent factors called parasporal inclusion bodies containing one or more insecticidal crystal proteins (ICPs) during their vegetative and/or sporulation growth phase. ICPs includes the more common Cry (crystal) proteins, the Cyt (cytolytic) proteins as well as the Vip (vegetative insecticidal) proteins" [12], "which are selectively toxic to different species of invertebrates upon ingestion. However, *Bt* has been highly explored as a microbial biopesticide for the control of insect pests especially in the Orders of Lepidoptera, Diptera and Coleoptera for over six decades" [13] due to its potent insecticidal activity, specificity against target insects, biodegradable nature and safety to non-target organisms [14]. Discovery of ICPs, isolation of *Bt* kurstaki (HD-1 strain) and development of effective formulation techniques were the key events, which helped *Bt* to evolve as a successful biopesticide and continues to hold 95 % of the 1% market share of biopesticides in the global pesticide market [15,16].

"Despite the effective application of *Bt* toxins for the biological control of pests, at present it is necessary to identify novel *Bt* isolates with promising Cry proteins for greater toxicity to counter the potential resistance evolved by insects" [17]. Hence, the present work aimed to perform molecular and morphological characterization of novel *Bt* isolate T352 toxic to *S. frugiperda* which might be exploited to develop new products and manage resistance in various agriculturally important insect pests.

2. MATERIALS AND METHODS

2.1 Insect culture

"Initial laboratory culture of *S. frugiperda* was obtained from NBAIR, Bangalore and maintained at *Bt* laboratory, Department of Plant Biotechnology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. The larval cultures were mass reared on modified CIMMYT diet" [18, 19]. After attaining second instar, the larvae were individually reared on vials up to pupal stage with sufficient amount of diet in order to prevent cannibalism. Pupae were transferred to rearing cages for adult emergence and oviposition. The adult moths were supplemented with 10% sugar solution containing vitamin E. After rearing for six generations at laboratory conditions, (25±1 ℃; 75±5 % RH; 16:8 light: dark hours) uniform insect population was used for bioassay.

2.2 *Bt* Isolates

An indigenous *Bt* isolate T352 was received from the *Bt* Laboratory, Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore along with positive standard strain HD-1 and negative acrystalliferous strain 4Q7. These cultures were revived and maintained on T3 medium (composition/ 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 to 7.0). Purified single colonies were obtained by quadrant streak plate technique.

2.3 Colony and Crystal Morphology

Colony morphology of indigenous *Bt* isolate T352 was examined in terms of colour, surface, elevation and margin by observing single bacterial colony from the culture plates maintained at 30 °C for 24 hrs. Single colony of

Bt culture was inoculated in 5 ml of T3 broth (mother culture) and incubated at 30 ℃ for 12 hrs at 200 rpm. A total of 250 µl mother culture (1%) was transferred into 25 ml of T3 broth and incubated for 48 hrs at 30 ℃ with 200 rpm. Subsequently, this 48 hrs grown bacterial culture was smeared on glass slide, heat fixed and stained with Coomassie brilliant blue (0.133% Coomassie Brilliant Blue G250 in 50% acetic acid) for 1 min in order to observe the parasporal crystalline inclusions under bright field microscope at 100X magnification (Euromex iScope).

2.4 Protein Profiling by SDS-PAGE Analysis

Spore crystal mixtures of indigenous *Bt* isolate T352 and reference strains were isolated by following the standard protocol [20]. "Cell lysis was assessed by staining and microscopic observation, after confirming 90% cell lysis, 48 hrs grown culture was centrifuged at 10000 rpm (Centrifuge 5810R, Eppendorf, Germany) for 10 min at 4 ℃. After discarding the supernatant, the resulting pellets were suspended in 25 ml of ice-cold Tris– EDTA buffer (Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM PMSF - phenyl methyl sulphonyl fluoride) and washed thrice with the same buffer and washed once with 0.5 mM NaCl. Finally, the Spore- crystal pellets were suspended in 500 µl of sterile distilled water containing 1 mM PMSF and stored at -20 °C for protein profiling and bioassay studies. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by standard protocol" [21] to characterize the Cry proteins by using 10 % separating gel and 4% stacking gel.

2.5 PCR Screening of *cry* and *vip* Genes

Total genomic DNA of *Bt* isolate T352 was isolated along with reference strains by following the standard protocol [22]. Quality of the isolated DNA assessed by agarose gel electrophoresis (0.8 %) and quantified with NanoDrop spectrophotometer (Genova Nano, Jenway). PCR was performed for lepidopteran toxic insecticidal protein encoding genes (*cry1, cry2, cry9* and *vip3A*) using gene specific family primers (Table 1) in master cycler (Eppendorf nexus Gx2) with a 20 µl reaction mixture containing 1 µL 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.

2.6 Bioassay

The protein concentration of spore-crystal mixture was estimated by Bradford method [28] with Bovine Serum Albumin (BSA) as standard using ELISA reader (Biotek – Powerwave XS). The *in-vitro* insect bioassay was performed with different concentrations (3.60, 2.70, 1.80, 0.90, 0.45, 0.22 and 0.112 µg/ml) of *Bt* isolate T352 against neonates of *S. frugiperda* by leaf disc surface coating method. Uniform stage young maize leaves were cut into ~2 × 2 cm size, and 20 µl 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 neonate 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 4Q7 was used as positive and negative control respectively, and absolute control was maintained with water. The larval mortality was observed at 72 hrs after treatment and finally probit analysis was done [29].

3. RESULTS AND DISCUSSION

3.1 Colony and Crystal Morphology

Bt isolate T352 was found to be creamy white in colour and had irregular shaped flat colonies with undulated margin. Kaviyapriya et al. [30] reported that the indigenous *Bt* isolate T29 toxic to *S. frugiperda* was found to have creamy white, circular shaped colonies with serrated margin. Similarly, Maheesha et al. [31] found the creamy white, fried egg type colonies with undulated margin and irregular shape in the indigenous *Bt* isolate (T350) toxic to *S. frugiperda.* Bright field microscopic observation at 100x magnification showed the presence of bipyramidal shape of crystals produced by *Bt* isolate T352. Manikandan et al. [26] observed the occurrence of bipyramidal and cuboidal shapes of inclusions in *Bt* isolate T30 whereas isolate T48 contains only bipyramidal shaped inclusions. While, Navya et al. [32] observed that *Bt* isolate RM11 effective against *Plutella xylostella* was found to possess three different shapes of crystals such as bipyramidal, spherical and cuboidal. These results suggested that the *Bt* isolates toxic to lepidopteran insects were found to have bipyramidal and cuboidal shape of crystal inclusions.

3.2 Protein Profiling by SDS-PAGE Analysis

SDS-PAGE analysis of spore crystal mixtures from T352 confirmed the presence of Cry1 and Cry2 proteins in the size of ~130 kDa and ~65 kDa as expressed in standard strain HD-1 (Fig. 1). Ramalakshmi et al. [20] found two major protein bands of~ 130 kDa and~ 65 kDa along with proteins of 95,43 and 30 kDa in the indigenous *Bt* isolates screened. Similarly, isolate GS4 tested by Patel and Ingle [33] against larvae of *Helicoverpa armigera,* showed multiple proteins in the range of 88, 54, 97, 175 and 135 kDa indicating the presence of more than one Cry protein and 12 *Bt* isolates effective against *Spodoptera littoralis* revealed the protein bands with different molecular weights in a range of 20-130 kDa [34]. Navya et al. [35] studied protein profile in 60 indigenous *Bt* isolates and observed that variation in the molecular weight of *Bt* protein ranging from 26 to 124 kDa in size.

Table 1. List of primer sequences used for gene profiling

Gene	Primer sequence	Product size	Reference
cry1	FP: 5'-CATGATTCATGCGGCAGATAAAC-3' RP: 5'-TTGTGACACTTCTGCTTCCCATT-3'	277 bp	[23]
cry1A	FP: 5'-GCCCCGGGCCTGGGTCAAAAATTGATATTTAG -3' RP: 5'-CGGGTCGACTAAATTGGATACTTGATCA -3'	2.1 Kb	[24]
cry1Ab	FP: 5'-CCCCGGGCCTGGGTCAAAAATTGATATTTAG-3' RP: 5'-GCTGCAGTGCTCTTTCTAAATCATATCTGCC-3'	2.1 Kb	[25]
cry2	FP: 5'-GTTATTCTTAATGCAGATGAATGGG-3' RP: 5'-CGGATAAAATAATCTGGGAAATAGT-3'	700 bp	[23]
cry2A	FP: 5'-ATGGTACCATGAATAATGTATTGAATAGTGGA-3' RP: 5'-GTTCTAGACTCAAACCTTAATAAAGTGGTG-3'	1.9 Kb	[26]
cry2Aa	FP: 5'-GTTATTCTTAATGCAGATGAATGGG -3' RP: 5'-GAGATTAGTCGCCCCTATGAG-3'	498 bp	[23]
cry2Ab	FP: 5'-GTTATTCTTAATGCAGATGAATGGG-3' RP: 5'-TGGCGTTAACAATGGGGGGAGAAAT-3'	546 bp	[23]
cry9	FP: 5'-CGGTGTTACTATTAGCGAGGGCGG-3' RP: 5'-GTTGAGCCGCTTCACAGCAATCC-3'	345 bp	[23]
vip3A	FP: 5'-CCTCTATGTTGAGTGATGTA-3' RP: 5'-CTATACTCCGCTTCACTTGA-3'	1.0 Kb	[27]

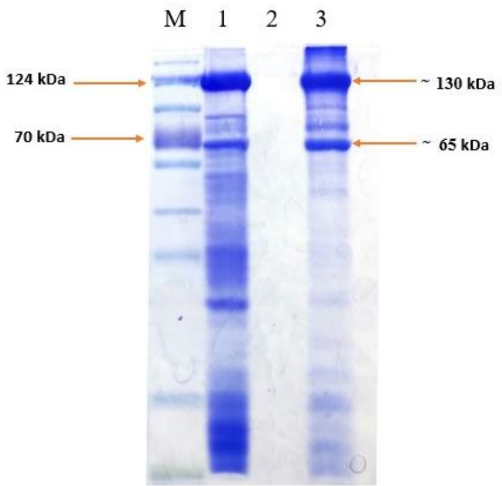

Fig. 1. Protein profiling by SDS-PAGE

Lane M-High range molecular marker; Lane 1- positive control, HD-1; Lane 2- negative control, 4Q7; Lane 3-T350

3.3 PCR screening of *cry* and *vip* genes

PCR screening for lepidopteran toxic gene families confirmed the presence of *cry1, cry1Ab, cry1Ac, cry2, cry2Aa, cry2Ab* and *vip3A* genes in isolate T352 (Table 2). Various studies on indigenous *Bt* isolates revealed the *cry1, cry1Aa, cry1Ab, cry1Ac, cry2Aa, cry2Ab* and *cry9* genes contributing the toxicity against major lepidopterous insects [24,35-38] and the dominance of *cry1* and *cry2* gene existing together was usually high [39]. The *Bt* isolate T352 having *cry1* and *cry 2* along with *vip3A* gene and toxic to *S. frugiperda* was in agreement with previous studies [40].

3.4 Bioassay against Fall Armyworm

The *in vitro* bioassay with 25 µg/ml of protein concentration against neonates of fall armyworm exhibited 100 % larval mortality in both isolate T352 and standard strain HD-1. Furthermore, isolate T352 recorded LC_{50} value of 1.927 µg/ml whereas HD-1 exhibited 0.421 µg/ml of LC_{50}. The significance of mortality data was confirmed by chi-square test (Table 3, Fig 2). Maheesha et al. [31] recorded LC_{50} value of 2.04 µg/ml in an indigenous *Bt* isolate against *S. frugiperda.* Similarly, *Bt* isolate RM11 expressed the toxicity on *Plutella xylostella* with 4.51 µg/ml [32]. Lone et al*.* [41] reported two isolates, JK12 and JK17 with LC_{50} value of 184.62 and 275.39 µg/ml against *Helicoverpa armigera.* Soares Figueiredo et al. [42] found that synergistic effect of Vip3 and Cry1 proteins expressed more toxicity on *S. frugiperda. Bt* strain 1644 with cuboidal crystal showed more toxicity against *S. frugiperda* than strain 344 with bipyramidal crystal [39]. Individual Cry proteins have lower toxicity as compared to the larvicidal activity of Cry proteins when administrated in combinations [43].

Table 2. PCR screening for *cry1*, *cry2* and *vip3* genes

Isolate/ strain	Genes						
	cry1	*cry1Ab*	*cry1Ac*	*cry2*	*cry2Aa*	*cry2Ab*	*vip3A*
HD- 1	+	+	+	+	+	+	+
4Q7	-	-	-	-	-	-	-
T352	+	+	+	+	+	+	+

+ PCR positive; - PCR negative

Table 3. Toxicity of indigenous *Bt* isolate T352 against *S. frugiperda*

Bt isolate/ Positive strain	LC50 (µg/ ml)	95% confident limits of concentration (µg/ ml)	Regression equation	χ2
T352	1.927	1.11 – 3.33	y = 4.74 + 0.89x	0.36
HD1	0.421	0.31 – 0.57	y = 5.54 +1.43x	1.11

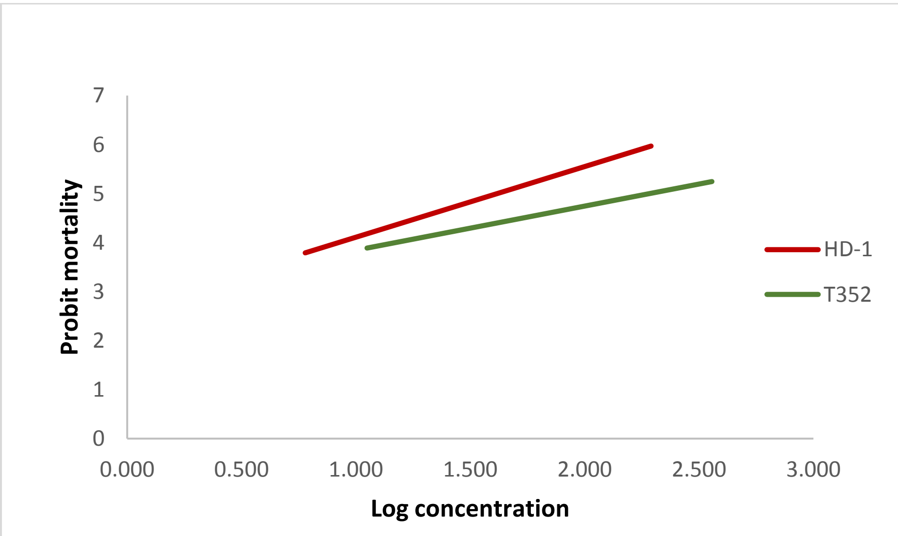

Fig. 2. Probit analysis for toxicity of *Bt* isolates against *S. frugiperda*

4. CONCLUSION

The higher level of toxicity of T352 well pronounced by the presence of cry1, cry2 and vip3A genes. Occurrence of different combination of pesticidal proteins delay the development of resistance in FAW population. The whole genome sequencing of *Bt* isolate T352 will be helpful in identifying the presence of novel cry genes. This present study suggested that the isolate T352 can be used for further formulation studies and the genes encoding pesticidal proteins may be cloned and if found to be effective, can be used for the development of transgenic plants against FAW and other lepidopteran pests.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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