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# Characterization of indigenous *Bacillus thuringiensis* isolate T350 toxic to fall armyworm, *Spodoptera frugiperda*

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**Abstract**
*Bacillus thuringiensis* is widely used in pest management as a biopesticide due to its effectiveness and specificity. Morphological, molecular and insecticidal characteristics of an indigenous *Bt* isolate T350 toxic to fall armyworm (FAW), *Spodoptera frugiperda* was studied. The *Bt* isolate T350 had creamy white, fried egg type colony which is irregular in shape with undulated margin. Bipyramidal shape of crystalline inclusions was found to present in isolate *Bt* isolate T350. The isolate produced protein bands of~130 kDa and~65 kDa size in SDS PAGE analysis and PCR screening confirmed the presence of *cry1*, *cry2* and *vip3* genes. $LC_{50}$ of the isolate was found to be 2.04 µg/ml against the neonate FAW, compared to 1.93 µg/ml for standard strain HD-1.

**Keywords:** fall armyworm, *Bacillus thuringiensis*, T350, SDS-PAGE, PCR screening

## 1. Introduction
Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is a serious agricultural pest which is known to attack more than 353 host plants (Montezano *et al*., 2018) [15]. This pest was originated from tropics and sub-tropics of Western hemisphere and invaded to Karnataka, India during May-June, 2018 (Sharanabasappa *et al*., 2018). In Tamil Nadu, its occurrence was first reported in November, 2018 at Erode and Karur districts (Srikanth *et al.*, 2018). Larval feeding causes severe damage, particularly to corn, with yield losses ranging from 17 to 72 percent depending on location and conditions (Murúa *et al*., 2015) [16]. Chemical insecticides are widely used to control this highly polyphagous pest (Young, 1979) [26] but their sole reliance may result in the development of resistance and environmental pollution (Yu, 1992; Belay *et al*., 2012) [3].
One of the successful alternatives to chemical pesticides is the entomopathogenic bacterium, *Bacillus thuringiensis* (*Bt*). The insecticidal activity of this bacterium is mainly due to the presence of crystalline proteins encoding insecticidal genes which are expressed during sporulation stage (Schnepf *et al*., 1998) [24]. Crystalline inclusions, when ingested by the larvae, solubilize in the midgut leading to the release of δ-endotoxins. The protoxins are then activated by midgut proteases enzyme and these activated toxins interact with midgut epithelium which cause disruption of membrane integrity leading to death (Gill *et al.*, 1992). *Bt* is diversified throughout the world, and each isolate produces toxins that are specific to a specific group of insect pests without affecting higher organisms or the environment (Schnepf *et al*., 1998) [24]. *Bt* toxins are used in the production of biopesticides and the genes encoding these proteins are used in the development of transgenic *Bt* plants to manage different group of insect pests (Romeis *et al*., 2006) [22]. Based on the preliminary toxicity assays, one of the locally isolated *Bt* isolate T350 was found to be toxic to *S. frugiperda*. The present investigation was performed to characterize the T350 isolate through SDS-PAGE analysis and PCR screening.

## 2. Materials and Methods
### 2.1 Insect culture and *Bt* isolates
Initial insect culture was obtained from FAW laboratory, Dept. of Agricultural Entomology and reared on artificial diet. An indigenous isolate T350, reference strain HD-1 and acrystalliferous *Bt* strain 4Q7 were received from the *Bt* Laboratory, Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore, India. After maintaining for six generations

on diet under laboratory conditions (25±1 ºC; 75±5% RH; 16:8 light: dark hours) uniform insect population was used for bioassay studies. Bt 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). All the experiments were performed at Department of Plant Biotechnology, TNAU.

### 2.2 Colony, crystal morphology and SDS-PAGE analysis

Colony morphology of indigenous *Bt* isolate T350 was observed visually, under low power stereo zoom microscope. Single purified colony of *Bt* isolate T350 was inoculated in 5 ml of T3 broth as mother culture and incubated at 30 ºC for 12 hours at 200 rpm. One per cent (250 µl) of mother culture was transferred into 25 ml of T3 broth and incubated for 48 hours at 30 ºC with 200 rpm. A loop of inoculum was taken and smeared on a sterile glass slide, heat fixed and stained with 0.133 % Coomassie brilliant blue dye. The crystalline inclusions were observed under bright field microscope at 100X magnification (Euromex iscope).

After 90 per cent lysis, culture was centrifuged at 10000 rpm for 10 min at 4 ºC and the pellets were resuspended 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) [19]. Protein profiling by SDS-PAGE analysis was performed by standard protocol (Laemmli, 1970) [12] to characterize the Cry proteins by using 10 % separating gel and 4% stacking gel.

### 2.3 PCR Screening for *cry* and *vip* genes

Genomic DNA isolated from T350 and standard strains (HD-1 and 4Q7), by standard protocol of Kalman *et al.* (1993) [11], was used for PCR screening in a 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 specific primers (Table 1).

**Table 1:** List of primer sequences used

| S. No. | Primer sequence | *cry* gene | Amplicon size | Reference |
|---|---|---|---|---|
| 1. | FP: 5′-CATGATTCATGCGGCAGATAAAC-3′<br>RP: 5′-TTGTGACACTTCTGCTTCCCATT-3′ | *cry1* | ~277 bp | Ben-Dov *et al.* (1997) [4] |
| 2. | FP: 5′-CCCCGGGCCTGGGTCAAAAATTGATATTTAG-3′<br>RP: 5′-GCTGCAGTGCTCTTTCTAAATCATATCTGCC-3′ | *cry1Ab* | ~2.1 Kb | Darsi *et al.* (2010) [5] |
| 3. | FP: 5′- GTATGCTTCTGTAACCCCGATTCACCTC-3′<br>RP: 5′-CCTGCAGTCCCACTAAAATTTCTAACACCTACTA-3′ | *cry1Ac* | ~195 bp | Alberola *et al.* (1999) [1] |
| 3. | FP: 5′-GTTATTCTTAATGCAGATGAATGGG-3′<br>RP: 5′-CGGATAAAATAATCTGGGAAATAGT-3′ | *cry2* | ~700 bp | Ben-Dov *et al.* (1997) [4] |
| 4. | FP: 5′-GTTATTCTTAATGCAGATGAATGGG -3′<br>RP: 5′-GAGATTAGTCGCCCCTATGAG-3′ | *cry2Aa* | ~498 bp | Ben-Dov *et al.* (1997) [4] |
| 5. | FP: 5′-GTTATTCTTAATGCAGATGAATGGG-3′<br>RP: 5′-TGGCGTTAACAATGGGGGGAGAAAT-3′ | *cry2Ab* | ~546 bp | Ben-Dov *et al.* (1997) [4] |
| 6. | FP: 5' CGGTGTTACTATTAGCGAGGGCGG 3'<br>RP: 5' GTTTGAGCCGCTTCACAGCAATCC 3' | *cry9* | ~350 bp | Ben-Dov *et al.* (1999) [4] |
| 7. | FP: 5′-CCTCTATGTTGAGTGATGTA-3′<br>RP: 5′-CTATACTCCGCTTCACTTGA-3′ | *vip3A* | ~1.0 Kb | Jain *et al.* (2012) [9] |

### 2.4 Bioassay

The *in-vitro* insect bioassay was performed with different concentrations of *Bt* isolate T350 against neonates of *S. frugiperda.* Young maize leaves (2nd leaf from top) were cut into 2x2 cm size and 20 µl of spore crystal mixture of different concentrations was coated on both sides of leaves (10 µl on each side) and air dried. The treated leaf was placed on a moist filter paper inside a plastic container of 3 cm diameter. The egg masses ready to hatch were placed on a Petri plate on the previous night and 12 hours old pre-starved neonate larvae were released @ 10 per treatment. HD-1, 4Q7 and water was used as positive, negative and absolute control. The larval mortality was observed after 72 hours of treatment and probit analysis was done (Srinivasan, 2004)

## 3. Results and Discussion

### 3.1 Colony and crystal morphology

*Bt* isolate T350 was found to be creamy white in color and had fried egg type colonies with undulated margin and irregular shape. Crystal morphology of *Bt* isolate T350 showed the presence of bipyramidal shaped crystals at 100X magnification. Previously, another indigenous isolate T29 which was also toxic to S. frugiperda was found to have creamy white, fried egg type and circular shaped colonies with serrate margin (Kaviyapriya *et al.*, 2019). Bt isolate UK-762D tested by Geeta *et al.* (2012) [7] had two types of crystals *viz.,* cuboidal and bipyramidal whereas three types of crystal shapes (bipyramidal, cuboidal and spherical) were reported by Ganesh *et al.* (2018) in Bt isolate T532.

### 3.2 Protein profiling by SDS Page

SDS-PAGE analysis of *Bt* isolate T350 confirmed the presence of Cry1 and Cry2 proteins of ~130 kDa and ~65 kDa size as in standard strain HD-1 (Fig. 1). Similarly, Salama *et al.* (2015) [23] tested the *Bt* isolates against *S. littoralis* and *Helicoverpa armigera* which produced protein bands of 127 and 130 kDa. Protein profiling by SDS-PAGE analysis showed the presence of different protein bands of 135, 95, 65, 43, and 30 kDa size in different isolates (Ramalakshmi and Udayasuriyan, 2010; Hernández *et al.*, 2005) [19, 8].

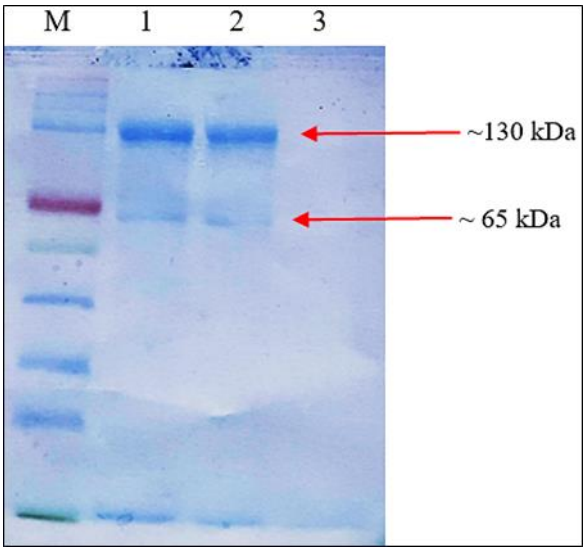


**Fig 1:** Protein profiling by SDS-PAGE

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

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

PCR analysis using lepidopteran toxic gene families showed that both isolate T350 and standard strain HD-1 were PCR positive for *cry1*, *cry1Ab*, *cry1Ac*, *cry2*, *cry2Aa*, *cry2Ab* and *vip3A* genes (Table 2). Previous studies from different laboratories showed that indigenous Bt isolates were PCR positive for *cry1*, *cry1Aa*, *cry1Ab*, *cry1Ac*, *cry2Aa*, *cry2Ab* and *cry9* genes (Ramalakshmi *et al.*, 2014; Reddy *et al.*, 2012; Manikandan *et al.*, 2016; Ganesh *et al.*, 2018; Reyaz *et al.*, 2020) [18, 20, 16].

**Table 2:** PCR screening for *cry1*, *cry2* and *vip3* genes (+ PCR positive; -PCR negative)

| Isolate/ strain | *Genes* | | | | | | |
|---|---|---|---|---|---|---|---|
| | **cry1** | **cry1Ab** | **cry1Ac** | **cry2** | **cry2Aa** | **cry2Ab** | **vip3A** |
| HD-1 | + | + | + | + | + | + | + |
| 4Q7 | - | - | - | - | - | - | - |
| T350 | + | + | + | + | + | + | + |

### 3.4 Bioassay

Initial bioassay with 25 µg/ml of protein concentration revealed that the isolate T350 and positive control HD-1 produced 100 per cent mortality against the neonates of *S. frugiperda*. In Probit analysis, isolate T350 recorded $LC_{50}$ value of 2.04 µg/ml as against 1.99 µg/ml in positive control HD-1 (Table 3; Fig. 2). Ammouneh *et al.* (2010) recorded $LC_{50}$ values ranging from 8.4 to 46.8 µg/g for 12 isolates against *Ephestia kuehniella*. Two *Bt* strains 344 and 1644 with bipyramidal and cuboidal shaped crystals, showed $LC_{50}$ values $8.21x10^6$ and $2.07x10^6$ spores/mL, respectively against *S. frugiperda* (Valicente and Luna, 2008) [25]. Lone *et al.* (2017) [13] recorded median lethal concentration of isolate JK12 as 184.62 µg/ml against *H. armigera*. Polanczyk *et al.* (2000) [17] recorded $LC_{50}$ values of $6.7x10^6$ and $8.6x10^6$ cells/ml, respectively against *S. frugiperda* in *Bt aizawai* HD 68 and *Bt* 4412 which. The *Bt* isolate SWK1 recorded $LC_{50}$ value of 2.448 µg/ml against *S. litura* (Reyaz *et al.*, 2017).
Characterization of *Bt* isolate T350 revealed the production of lepidopteran specific Cry1 and Cry2 proteins, with $LC_{50}$ value of 2.04 µg/ml which is on par with standard strain HD-1 (1.99 µg/ml). Hence, the isolate T350 can be used for further formulation studies and the genes encoding Cry proteins may be further cloned and if found to be novel, can be used for development of transgenic plants against FAW and other lepidopteran pests.

**Table 3:** Toxicity of indigenous *Bt* isolate T350 against FAW

| ***Bt* isolate** | **$LC_{50}$ (µg $ml^{-1}$)** | **95% confident limits of concentration (µg $ml^{-1}$)** | **Regression equation** | **$\chi 2$** |
|---|---|---|---|---|
| T350 | 2.04 | 1.65 - 2.51 | y = 8.388 + 2.419 x | 2.88 |
| HD1 | 1.99 | 1.46 – 2.72 | y = 7.455 + 1.754 x | 3.56 |

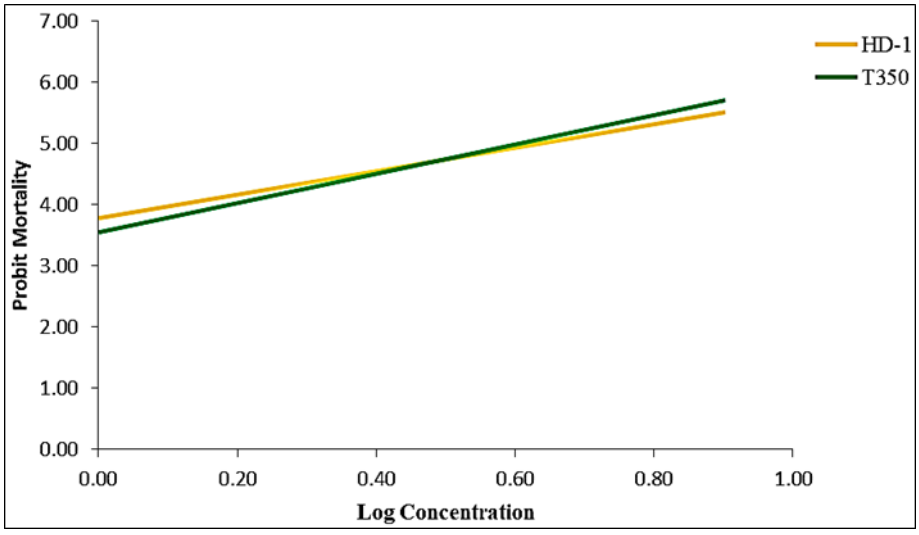


**Fig 2:** Probit analysis for toxicity of *Bt* isolates against *S. frugiperda*

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