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Characterization of a *Bt* isolate derived from naturally infected fall armyworm, *Spodoptera frugiperda* (JE Smith) larvae in maize field

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Abstract
Fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Noctuidae, Lepidoptera) causes economic losses in maize in India, since 2018. Microbial control, especially *Bacillus thuringiensis* (*Bt*), plays an important role in integrated pest management programme. During the survey of FAW in maize, *Bt* infected FAW larvae were collected in K. Alangulam village, Kuruvikulam block, Tenkasi district, Tamil Nadu, India and brought to the laboratory. *Bt* was isolated from the infected larvae and cultured in T_3 medium. The colonies were circular in shape with serrated margin and creamy white in colour. The colonies had umbonate elevation and produced cuboidal shaped crystals. While screening the *Bt* isolate with lepidopteran toxic *cry* genes *i.e.*, *cry1*, *cry2Aa*, *cry2Ab*, *cry9* and *vip3A* genes by PCR, it showed the presence of *cry2Aa* gene. SDS PAGE analysis showed the presence of Cry2 crystal protein with the molecular weight of ~65 kDa. Bioassay studies with the spore-crystal mixture of the isolate exhibited 70 per cent mortality against maize fall armyworm, *S. frugiperda*.

Keywords: Fall armyworm, *Bacillus thuringiensis*, PCR, SDS-PAGE, bioassay

1. Introduction
Maize is one of the important cereal crops in India, ranks third in production next to wheat and paddy, which contributes about 9% in staple human food and nearly Rs.100 billion in agricultural GDP [7]. In 2018-19, total area covered under maize cultivation reached 9.2 million ha with average production and productivity of 27.8 million MT and 2965 kg/ha respectively, in India [12]. In November 2019, India exported around 40,720 MT of maize [20]. Maize acts as a good source of high fibre content, antioxidants, vitamins and minerals. It has been directly used for human consumption or industrially processed food and quality livestock feed especially for poultry birds. It has a rich source of energy and high digestibility capacity for poultry. More than 50 per cent of the total maize production is utilized as quality feed in poultry sectors [29].

Though it is reported that there are about 139 insect-pests causing damage to maize in both field and storage conditions [8], farmers did not face much challenges in maize cultivation especially due to the infestation by insect pests until 2017 in India. After the invasion of the exotic insect pest, fall armyworm (FAW), *Spodoptera frugiperda* (JE Smith), (Noctuidae, Lepidoptera), problems are encountered in maize cultivation as it causes much economic damage and serious loss to the farmers. The origin of FAW belongs to tropical and subtropical America, invaded Africa in 2016 and India in 2018. A gravid female can lay up to 1500-2000 eggs during its lifetime [5] and its life cycle is very short up to a period of 30-45 days . It is a voracious feeder which feeds on leaves, stem, and reproductive parts of more than 80 plant species. In India, it was first reported on maize in May 2018 in the state of Karnataka and subsequently spread all over the country. It can complete several generations in a single season and the population reaches economic injury level (EIL) within a short period of time [31]. The ideal climatic conditions and availability of suitable host plants lead FAW to become an endemic pest in India. Hence, management of this pest is important to sustain the yield in maize.

Microbial control measures play an important role as one of the components in IPM programme. Microbial agents such as bacteria, fungi, and virus act as an alternative to chemical insecticides and give promising control for insect pests. Among the entomopathogenic bacteria, *Bacillus thuringiensis* (*Bt*) Berliner is more effectively used in

controlling insect pests. *Bt* is an aerobic, spore-forming bacterium that produces parasporal crystalline proteins known as δ-endotoxins [22]. These crystal toxins when entered into the insect midgut get activated in alkaline pH and bind with the brush border epithelium. It creates lytic pores on midgut epithelium which leads to osmotic-cell lysis and septicaemia in insects.

In this present study, a new *Bt* isolate was isolated from naturally infected *S. frugiperda* larvae and carried out morphological and molecular characterization and toxicity analysis against *S. frugiperda*. This will be helpful for identification of a novel *Bt* strain for the management of *S. frugiperda.*

2. Materials and methods

2.1 Collection of *Bt* infected *S. frugiperda* larvae

Bt infected larvae were collected during the survey on 26.11.2019 at K. Alangulam village, Kuruvikulam block, Tenkasi district, Tamil Nadu (Latitude: 8° 52' 12.00" N; Longitude: 77° 30' 0.00" E) and brought to Department of Agricultural Entomology, Agricultural College and Research Institute, Killikulam, Thoothukudi district, Tamil Nadu.

2.2 Isolation of *Bt* from infected larvae

The *Bt* infected larvae (Fig. 1A) were surface sterilized with 0.5% sodium hypochlorite solution for two minutes and washed with sterilized water and dried [24]. The sterilized larvae were inoculated into T_3 medium and incubated at 30 °C for 24 hours (Fig. 1B). Then a loopful of *Bt* culture (developed from the infected larvae) was taken and streaked on T_3 medium plates for further morphological and molecular studies. The isolate was named as ALM1.

2.3 Morphological characterization of *Bt* isolate

The individual colonies were visually observed to study colony morphology. Crystal morphology was studied by observing the spore-crystal mixture which was harvested as per the protocol [25]. A loopful of *Bt* culture was inoculated into 5 ml T_3 broth and incubated at 30 ^{0}C and 180 rpm overnight. After 12-14 hours of incubation, 250 µl of culture was transferred to 25 ml T_3 broth and incubated at 30 ^{0}C and 180 rpm for 48 hours. After that, a drop of culture was taken and smeared on a sterile glass slide, stained with 100µl of Coomassie Brilliant blue G250 stain, observed under 100x magnification in bright field microscope for studying crystal morphology.

2.4 Extraction of spore-crystal mixture from the *Bt* isolate

A loopful of pure culture was inoculated into the 5 ml T_3 broth and incubated at 30 ^{0}C and 180 rpm. After 12-14 hours of incubation, 250 µl of culture was transferred to 25 ml T_3 broth and incubated at 30 ^{0}C and 180 rpm for 48 hours. Then the culture was observed for cell lysis stage. The spore-crystal mixture or protein was harvested when the culture attained 90% lysis stage. The sporulated culture was transferred to 4 ^{0}C for at least half an hour and then centrifuged at 10,000 rpm for 10 minutes. The attained pellet was washed thrice with 25 ml of ice-cold Tris-EDTA buffer (10 mM Tris, 1 mM EDTA with 1 mM PMSF, pH 8.0) which contained PMSF (Phenyl methyl sulfonyl fluoride) and once with 25 ml of ice cold 0.5 M NaCl solution. At the end, pellet was suspended in 100 µl of sterile water along with 1mM PMSF and stored at -20 ^{0}C [25].

2.5 Molecular characterization of *Bt* isolate

2.5.1 Genomic DNA isolation

Genomic DNA was isolated as per the protocol [14]. The isolated DNA was quantified by loading on 0.8% agarose gel and analyzed through agarose gel electrophoresis [27].

2.5.2 PCR screening for lepidopteran toxic *cry* genes

Screening of the *Bt* isolate (ALM1) for lepidopteran toxic *cry* genes *viz.*, *cry1*, *cry2Aa*, *cry2Ab*, *cry9* and *vip3A* gene was performed by PCR [3,4] using specific primers (Table 1) in Thermocycler (Eppendorf Master Cycler Personal, Germany). HD1 was used as reference strain for *cry1*, *cry2Aa*, *cry2Ab* and *vip3A* genes and 4G1 for *cry9* gene. The reference strains were obtained from Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, Tamil Nadu, India. A total volume of 25µl reaction mixture containing 12.5µl of master mix, 2 µl each of forward and reverse primers, 3 µl of genomic DNA and 5.5 µl of sterile water and the reaction was carried out for 30 cycles. Temperature profile is given in Table 2.

2.5.3 Protein profiling through SDS-PAGE analysis

The extracted protein (spore-crystal mixture) of the *Bt* isolate, ALM1 was used and SDS-PAGE analysis was performed to analyze the protein profile by using 10% separating gel and 4% stacking gel [16]. The molecular mass of protein was identified by means of a higher range protein molecular weight marker (GeNeiTM, Bengaluru, India). The gels were stained with 0.4% Coomassie Brilliant Blue R250 and destained with de staining solution comprising glacial acetic acid, methanol and distilled water in the ratio of 1:4:5 v/v.

2.6 Toxicity analysis of *Bt* isolate upon *S. frugiperda* larvae

Bioassay was conducted to determine the toxic nature of the newly isolated *Bt* strain against neonates of *S. frugiperda*. The study was conducted based on the artificial diet contamination method. The artificial diet used in this present study was prepared [23], made in to small uniform pieces (cakes) and all the sides of the solidified diet cakes were contaminated with 30 µl of crude spore-crystal mixture harvested from the isolate. The diet smeared with HD1 (reference strain) and untreated diet cakes were taken as positive and negative control, respectively. Fifty neonate larvae were used for each treatment. Individual larva was released in separate plastic container containing the treated/untreated artificial diet cake. The observations were made periodically on every 24 hours up to 7 days to assess the per cent mortality.

3. Results

3.1 Morphological characterization of the *Bt* isolate (ALM1)

3.1.1 Colony morphology

The newly derived *Bt* isolate (ALM1) was sub-cultured on T_3 medium for single colony isolation. After 24 hours of inoculation, the isolate produced creamy white colonies. These colonies were circular and fried egg type with raised appearance, serrated margin and umbonate elevation (Fig. 1C).

3.1.2 Crystal morphology

Bt produces parasporal crystal inclusions during sporulation which differentiates it from remaining *Bacillus* spp. The new

Bt isolate (ALM1) produced cuboidal shaped crystal inclusions (Fig. 2) which were observed under bright field microscope.

3.2 Molecular characterization of *Bt* isolate

3.2.1 PCR screening for lepidopteran toxic *cry*/*vip* genes

PCR screening was done to see the presence of lepidopteran toxic *cry/vip* genes *viz.*, *cry1*, *cry2Aa*, *cry2Ab*, *cry9* and *vip3A* gene in the new isolate, ALM1. The results showed a band size of approximately 500bp and indicated the presence of *cry2Aa* gene alone (Fig. 3).

3.2.2 SDS-PAGE analysis

SDS-PAGE analysis was carried out with the proteins harvested from the isolate which exhibited a protein band with the molecular mass of ~ 65 kDa corresponding to Cry2 proteins similar to HD1 reference strain (Fig. 4).

3.3 Toxicity analysis against *S. frugiperda*

A bioassay was performed to identify the insecticidal nature of the new *Bt* isolate against the neonates of *S. frugiperda* under laboratory conditions through diet contamination method. The observations showed that the isolate exhibited seventy per cent mortality on neonates of *S. frugiperda*, while HD1 showed hundred per cent mortality and negative control had no mortality (Fig. 5).

4. Discussion

Bt is a naturally occurring spore-forming ubiquitous bacterium, found in various environments such as soil, leaf, insect cadaver, seed dust and spider web [21,2,25,32,26]. The usage of *Bt* based biopesticides has increased recently to counteract the resistance developed by insects for various synthetic insecticides. In this present study, a new *Bt* isolate (ALM1) was isolated from naturally infected *S. frugiperda* larva, identified the presence of lepidopteran toxic *cry*/*vip* genes and studied toxicity of the isolate to the maize fall armyworm, *S. frugiperda.*

4.1 Morphological characterization of *Bt* isolate

The study of colony and crystal morphology helps to differentiate *Bt* colonies from other *Bacillus* species. The *Bt* isolate (ALM1) produced creamy white, circular-shaped colonies with umbonate elevation and serrated margin. It is in conformity with the results of Chatterjee *et al.* [6], they isolated 11 types of *Bt* isolates from rice field which produced circular, white colonies. Lenina *et al.* [17] also found that *Bt* colonies produced creamy white rough colonies in their studies. Since there is an association between the crystal shapes and toxicity [19], scrutiny of crystal morphology is very much important. After 90% of cell lysis, crystals were observed under bright field microscope which showed cuboidal shaped crystals. Similar result was reported by Swiecicka *et al.* [30] during their studies. They found out that the relative predominance of cuboidal shaped crystals over bipyramidal crystals in their *Bt* strains isolated from soil samples. Ramalakshmi *et al.* [25] also observed that 27% of 316 isolates obtained from soil samples of Western Ghats produced cuboidal shaped crystals followed by 21% of bipyramidal crystals. Jain *et al.* [13] reported five different types of crystals *viz.*, bipyramidal, cuboidal, spherical, rectangular and irregular shaped crystals in their studies.

4.2 Molecular characterization of *Bt* isolate

PCR screening with *cry*/*vip* gene specific primers helps to see the distribution of lepidopteran toxic genes in *Bt* isolates. The results showed that the new *Bt* isolate (ALM1) isolate harboured only *cry2Aa* gene with an amplicon size of 500bp. Wang *et al.* [33] stated that 70% of isolates derived from various sources of China contain *cry2* gene. Sauka *et al.* [28] reported that 56 isolates (94.9%) possessed the combinations of both *cry2Aa* and *cry2Ab* genes, two isolates possessed *cry2Ab* gene (3.4%) and one isolate with *cry2Aa* gene (1.7%), out of 59 isolates tested. Liang *et al.* [18] reported that out of 791 *Bt* strains, 322 strains harboured *cry2* gene *viz.*,6.8% possessed *cry2Aa* gene followed by 2.5% by *cry2Ab*. The remaining isolates possessed both *cry2Aa* and *cry2Ab* gene (90.4%).

4.3 Protein profiling of insecticidal crystal protein

The insecticidal crystal proteins were harvested from the isolate and examined for their molecular mass through SDS-PAGE analysis**.** The analysis showed protein bands of size ~ 65 kDa which is similar to Cry2 proteins of HD1 reference strain. Zothansanga *et al.* [34] studied 82 *Bt* isolates from Jhum soils of Mizoram and reported that the isolates exhibited eleven different protein patterns. Among them, 18.69% showed protein bands similar to Cry2 proteins with the size of ~ 65 kDa. Reyaz *et al.* [26] examined 23 *Bt* strains obtained from various samples of Kashmir valley and found out that 73.92% strains produced protein bands ranging between 60-70 kDa which is similar to Cry2 and Cry3 proteins.

4.4 Bioassay

The insecticidal activity of the new isolate (ALM1) was analysed through preliminary bioassay studies using artificial diet coated with spore-crystal mixture. The isolate exhibited seventy per cent mortality on neonates of *S. frugiperda* larvae under laboratory conditions. These results are correlated with the findings of Alvarez *et al.* [1]. They analyzed the toxicity for fourteen *Bt* isolates isolated from diseased larvae and soil samples of northwest Argentina against *S. frugiperda* and observed that three isolates *viz.*, *Bt* RT, *Bt* LQ, and *Bt* LSM showed 53 to 100% mortality. Dos Santos *et al.* [10] assessed insecticidal nature of one hundred strains against *S. frugiperda*. Of these, five strains namely BR9, BR37, BR45 S608 and S1905 showed higher mortality when compared to other isolates. Kaviyapriya *et al.* [15] assessed the toxicity of an indigenous *Bt* isolate T29 through preliminary bioassay against *S. frugiperda* which showed 100% mortality on the third day of assay. Del Valle Loto *et al.* [9] characterized the toxic nature of native strain *Bt* RT obtained from Argentina and reference strain *Bt* HD1 formulations against third instar larvae of *S. frugiperda* biotypes and identified that the native strain had more potential than the standard strain.

5. Conclusion

At present, maize fall armyworm is a serious pest problem in India and causes a huge economic loss in maize cultivation. Identification of a biopesticide, like *Bt*, is much needed to avoid solely depending on chemical insecticides and also the consequences of indiscriminate usage of chemical insecticides. Characterization and screening of *Bt* isolates is a continuous effort in identification of effective *Bt* isolates. In this study, a new *Bt* isolate was obtained from naturally

infected larvae, characterized morphologically and molecularly and analyzed the toxicity against *S. frugiperda*, which are the pre-requisites for development of *Bt* formulation(s) or deployment of the gene(s) in development of transgenic plants.

Table 1: Details of primers used for screening of *Bt* isolate for lepidopteran toxic *cry/vip* genes

S. No.	Primer sequence (5' to 3')	*cry/vip* genes	Amplicon size	Reference
1.	FP: CATGATTCATGCGGCAGATAAAC RP: TTGTGACACTTCTGCTTCCCATT	*cry1*	~280 bp	Ben-Dov *et al.* [4]
2.	FP: GTTATTCTTAATGCAGATGAATGGG RP: GAGATTAGTCGCCCCTATGAG	*cry2Aa*	~500 bp	
3.	FP: GTTATTCTTAATGCAGATGAATGGG RP: TGGCGTTAACAATGGGGGGAGAAAT	*cry2Ab*	~550 bp	
4.	FP: CGGTGTTACTATTAGCGAGGGCGG RP: GTTTGAGCCGCTTCACAGCAATCC	*cry9*	~350 bp	Ben-Dov *et al.* [3]
5.	FP: ACATCCTCCCTACACTTTCTAATAC RP: TCTTCTATGGACCCGTTCTCTAC	*vip3A*	~680 bp	Espinasse *et al.* [11]

Table 2: PCR temperature profile for screening of *Bt* isolate for *cry1*, *cry2Aa*, *cry2Ab*, *cry9* and *vip3A* genes

S. No.	Step	Temperature profile and time				
		cry1	***cry2Aa***	***cry2Ab***	***cry9***	***vip3A***
1.	Initial denaturation	94 °C for 2 minutes				
2.	Denaturation	94 °C for 1 minute				
3.	Annealing	62 °C for 40 seconds	60 °C for 40 seconds		58 °C for 40 seconds	50 °C for 40 seconds
4.	Initial extension	72 °C for 1 minute				
5.	Step 2 to 4	30 cycles				
6.	Final extension	72 °C for 7 minutes				

A. B. C.

Fig 1: A) *Bt* infected larva, B) *Bt* culture from infected larva in T_3 medium, C) Individual *Bt* colonies

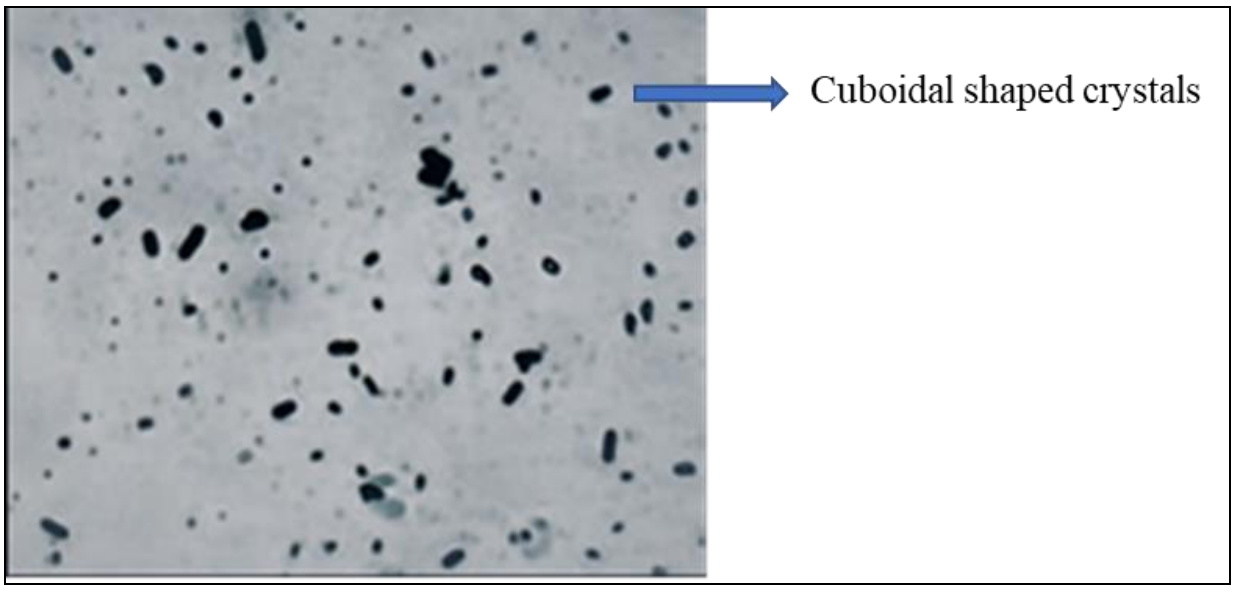

Fig 2: Crystal morphology

Fig 3: Amplification of *cry2Aa* gene in the *Bt* isolate by PCR HD1: Reference strain; WC: Water control; 1&2: Colonies from *Bt* isolate (ALM1)

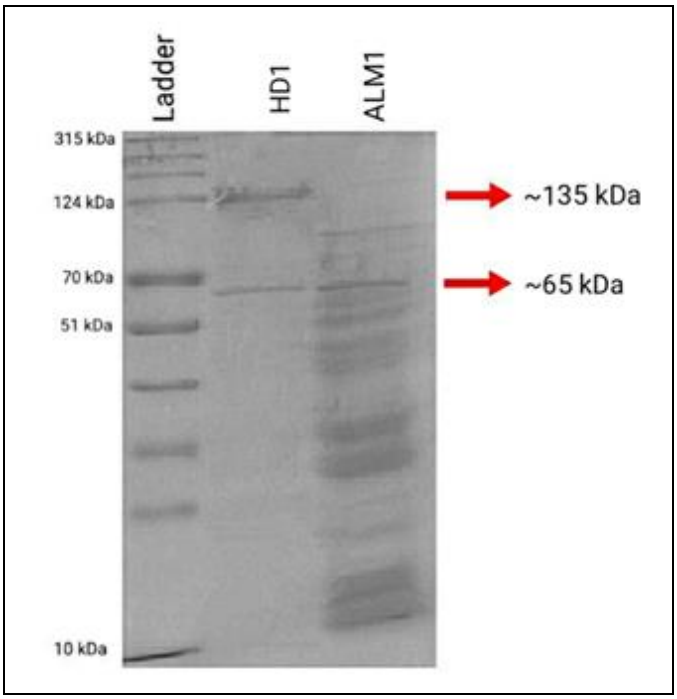

Fig 4: SDS-PAGE analysis of spore-crystal suspension of the *Bt* isolate (ALM1)

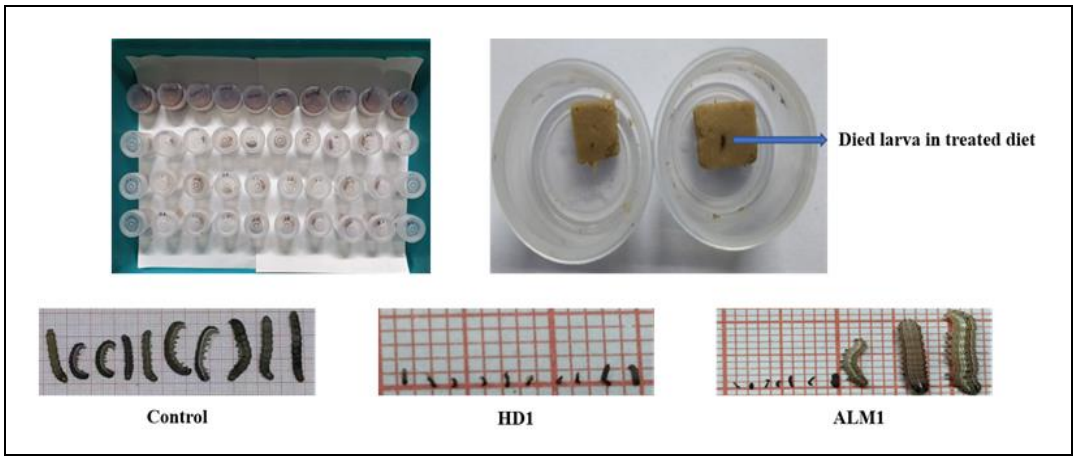

Fig 5: Artificial diet surface contaminated bioassay against *S. frugiperda* using the *Bt* isolate (ALM1)

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