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Characterization of indigenous *Bacillus thuringiensis* (*Bt*) isolates and screening for lepidopteran toxic insecticidal genes

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Abstract
Bacillus thuringiensis (*Bt*) is a widely distributed Gram-positive bacterium known for its ability to produce insecticidal toxins with high specificity for certain insect pests. In this study, 15 indigenous *Bt* isolates were characterized and screened for lepidopteran toxic insecticidal genes through PCR. The colour, surface, shape, elevation and margin of the isolates were studied. Diversified crystal shapes were observed among the isolates tested. PCR analysis confirmed the presence of *cry1, cry2* and *vip3A* genes in the isolates, either individually or in combination, while none of the isolates possessed *cry9* gene.

Keywords: *Bt* isolates, characterization, screening, PCR analysis

Introduction
India is an agriculture-based country and agriculture sector is one of the important sectors in Indian economy. The net cropped area in India is high (next to USA and China) and India ranks second in farm outputs, worldwide. Despite advanced technologies available in the field of agriculture, the production and productivity of agricultural and horticultural crops are highly affected by biotic and abiotic stresses. Food security has become a great challenge. To feed the alarming rate of increasing human population, agriculture has to be intensified with the available cropping area which is shrinking year by year because of urbanization and drought. Production has to be doubled in the developing countries to meet out the food need by 2050 [1]. Among the biotic stresses, insect pests play a major role in causing crop losses. The yield loss due to the insect pests alone accounts about 15.7 per cent [2].

Application of synthetic insecticides is the most commonly adopted method for insect pest management. Indiscriminate and continuous usage of synthetic insecticides led to the development of insecticide resistance in insects besides causing environmental damage and health hazards. Biopesticides are the alternatives to avoid these risks posed by the chemical insecticides. *Bacillus thuringiensis* (*Bt*) is the most effective insect pathogenic bacterium used for pest management, accounting for 2% of the overall insecticidal market. It is an aerobic spore forming, Gram-positive, soil-dwelling bacteria known for its ability to produce insecticidal proteins (crystalline inclusions) known as δ-endotoxin (delta-endotoxin) during sporulation. These toxins damage the midgut of the pest causing septicaemia and death within 2-3 days [3, 4]. These Cry proteins are innoxious to humans, vertebrates, plants and are completely biodegradable. *Bt* has a wide array of insecticidal activity against insect pests belonging to the major orders *viz.*, Lepidoptera, Diptera and Coleoptera. The *Bt* toxins are widely applied in the form of biopesticides or developing insect-resistant transgenic crops by introducing the toxin-encoding genes through genetic engineering approach. Exploring new *Bt* strains with different toxins should be continuous process to identify a novel *Bt* isolate. Hence, the *Bt* isolates have to be characterized and screened for their insecticidal genes to find out their potential against the insect pests. In this present investigation, fifteen isolates have been taken, morphologically characterized and screened for lepidopteran toxic insecticidal genes.

Materials and Methods
Bt culture
A total of 15 indigenous *Bt* isolates (T15, T29, T147, T148, T149, T150, T151, T152, T153, T154, T155, T157, T355, T527 and T543) with the reference strains HD-1 (positive for *cry1*,

cry2 and *vip3A* genes), 4AT0 (positive for *cry9* gene) and an acrystalliferous *Bt* strain 4Q7 (negative control), were obtained from the Bt Laboratory, Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore, India. These *Bt* isolates were revived and maintained on T3 medium (3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 6.9 g sodium dihydrogen phosphate, 8.9 g disodium hydrogen phosphate, 100 µl of 0.05 g manganese chloride dissolved in 1 ml of water, 20 g of Agar and made up to 1 litre using sterile water and the pH of the medium was adjusted within the range of 6.8 – 7.0) for its substantial growth.

Colony morphology

Single colonies of the isolates cultured in the Petri plates were visualized under a bright-field stereozoom microscope and various parameters such as colour, surface, shape, elevation and margin were studied.

Crystal morphology

A loopful of *Bt* culture taken from a single colony was inoculated into 5 ml of T3 broth as a mother culture and incubated at 30 °C for 12 hours at 200 rpm. One percent (250 µl) of the mother culture was transferred to 25 ml of T3 broth and incubated for 48 hours at 30° with 200 rpm. Thereafter, a loopful of bacterial culture was smeared on a sterile glass slide, heat fixed, and stained with 0.133% Coomassie brilliant blue dye (G250) for 1 minute. The stained slides were washed gently, blot dried and observed under a bright-field microscope at 100 X magnification to check the presence of parasporal crystalline inclusions (iScope, Euromex).

Genomic DNA isolation and PCR screening

Genomic DNA from the *Bt* isolates was extracted as per the protocol [5]. Extracted genomic DNA (30 ng) was subjected for PCR screening using specific primers to find out the presence of lepidopteran toxic insecticidal genes *viz.*, *cry1*, *cry2*, *cry9* and *vip3A* genes (Table 1). A total reaction volume of 20 µl containing 1 µl of template DNA, 1 µM of each primer (forward and reverse), 10 µl of PCR master mix (Smart Prime 2X) consisting of dNTPs, Taq polymerase, and 7 µl of nuclease-free water. The temperature profile of the PCR for screening the *Bt* isolates for the presence of *cry1*, *cry2* and *vip3A* genes are given (Table 2). PCR analysis was performed in Mastercycler (Nexus GX2, Eppendorf, Germany) and the PCR products were resolved in agarose gel electrophoresis with ethidium bromide (EtBr) staining. The amplified products were visualized under a UV trans-illuminator and documented (Bio-Print, Vilber).

Results and Discussion

Colony and crystal morphology of the *Bt* isolates

Majority of the tested isolates exhibited colonies with characteristics such as a creamy white colour, a surface resembling a fried egg, circular shape, raised elevation, and an entire margin. Colony surface found to be fried egg type for eight isolates, and the remaining seven were mucoid type. In the case of colony shape, 12 isolates had circular shapes and the remaining three isolates had irregular shapes. The colony elevation of eight isolates appeared flatand raised appearance in seven isolates. Out of 15 isolates, 10 isolates had the entire margin and the remaining five isolates had undulated margins (Table 3). Kaviyapriya *et al.* (2019) observed creamy white in colour with a fried egg surface, circular shaped with serrate margin in the *Bt* isolates screened [6]. Similarly, Navya *et al.* (2021) observed creamy white to off-white colour colonies with fried egg appearance, irregular shape, flat elevation and undulated margin from the *Bt* isolates screened [7]. In earlier days, the *Bt* isolates were identified mainly based on the presence of parasporal crystalline inclusions. Out of 15 isolates investigated in this study, the shape of the crystal inclusion varied *viz.*, cuboidal, spherical and bipyramidal (Fig.1). It is in line with the finding of Gothandaraman *et al.* (2022) [8].

PCR screening of indigenous *Bt* isolates for lepidopteran toxic *cry* and *vip* genes

PCR analysis of 15 Indigenous *Bt* isolates revealed that the *Bt* isolates had genes either alone or in combination of *cry* or *vip* genes. The isolates T152, T15, T29, T355, T527 and T543 had *cry1* gene. T152 isolate had *cry1* gene only. The isolates T15, T29, T355, T527 and T543 had *cry2* gene only. Five isolates, T15, T29, T355, T527 and T543 had both *cry1* and *cry2* genes. The *vip3A* gene was present in three isolates namely, T29, T355 and T527. Three *Bt* isolates, namely, T29, T355 and T527 were positive for *cry1*, *cry2* and *vip3A* genes together (Table 4). These findings are in comparable with earlier studies indicating the presence of *cry* and *vip* genes in *Bt* isolates in combination [9-12]. The findings [13] indicated that the *Bt* isolates carrying *vip3A* gene possess *cry1* and *cry2* genes, which shows similarity with our findings.

Table 1: Details of primers used for screening the *Bt* isolates

S. No.	Gene	Primer sequence	Product size	Reference
1.	*cry1*	FP: 5′-CATGATTCATGCGGCAGATAAAC-3	~ 277 bp	(Ben-Dov *et al.*, 1997) [6]
		RP: 5′-TTGTGACACTTCTGCTTCCCATT-3′		
2.	*cry2*	FP: 5′-GTTATTCTTAATGCAGATGAATGGG-3′	~ 700 bp	
		RP: 5′-CGGATAAAATAATCTGGGAAATAGT-3′		
3.	*cry9*	FP: 5′-CGGTGTTACTATTAGCGAGGGCGG-3′	~ 345 bp	
		RP: 5′-GTTGAGCCGCTTCACAGCAATCC-3′		
4.	*vip3A*	FP: 5′-CCTCTATGTTGAGTGATGTA-3′	~ 1.0 Kb	(Jain *et al.*, 2012) [7]
		RP: 5′-CTATACTCCGCTTCACTTGA-3′		

Table 2: PCR conditions for screening of *Bt* isolates for lepidopteran toxic insecticidal genes

S. No.	Step	Temperature profile and time			
		cry1	***cry2***	***cry9***	***vip3A***
1.	Initial denaturation	94 °C for 2 minutes			94 °C for 5 minutes
2.	Denaturation	94 °C for 40 seconds			94 °C for 1 minute
3.	Annealing	62 °C for 40 seconds	60 °C for 40 seconds	58 °C for 40 seconds	55 °C for 1 minute
4.	Extension	72 °C for 1 minute	72 °C for 40 seconds	72 °C for 1 minute	72 °C for 40 seconds
5.	Step 2 to 4	30 cycles			35 cycles
6.	Final extension	72 °C for 10 minutes			

Table 3: Colony morphology of *Bt* isolates

S. No.	*Bt* isolate	Colony morphology				
		Colour	**Surface**	**Shape**	**Elevation**	**Margin**
1.	T15	Off white	Fried egg	Irregular	Flat	Undulate
2.	T29	Creamy white	Fried egg	Circular	Flat	Undulate
3.	T147	Creamy white	Mucoid	Circular	Raised	Entire
4.	T148	Creamy white	Mucoid	Circular	Raised	Entire
5.	T149	Creamy white	Mucoid	Circular	Raised	Entire
6.	T150	Creamy white	Mucoid	Circular	Raised	Entire
7.	T151	Creamy white	Mucoid	Circular	Raised	Entire
8.	T152	Off white	Fried egg	Irregular	Flat	Undulate
9.	T153	Creamy white	Fried egg	Circular	Raised	Entire
10.	T154	Creamy white	Mucoid	Irregular	Flat	Undulate
11.	T155	Creamy white	Mucoid	Circular	Raised	Entire
12.	T157	Creamy white	Fried egg	Circular	Raised	Entire
13.	T355	Creamy white	Fried egg	Circular	Flat	Undulate
14.	T527	Creamy white	Fried egg	Circular	Flat	Entire
15.	T543	Creamy white	Fried egg	Circular	Flat	Entire
16.	HD 1	Off white	Fried egg	Irregular	Flat	Undulate

Table 4: PCR Screening of *Bt* isolates for lepidopteran toxic insecticidal genes

S. No.	*Bt* isolate/Reference strain	Lepidopteran toxic genes			
		cry1	***cry2***	***cry9***	***vip3A***
1.	T15	+	+	-	-
2.	T29	+	+	-	+
3.	T147	-	-	-	-
4.	T148	-	-	-	-
5.	T149	-	-	-	-
6.	T150	-	-	-	-
7.	T151	-	-	-	-
8.	T152	+	-	-	-
9.	T153	-	-	-	-
10.	T154	-	-	-	-
11.	T155	-	-	-	-
12.	T157	-	-	-	-
13.	T355	+	+	-	+
14.	T527	+	+	-	+
15.	T543	+	+	-	-
16.	HD1(Reference strain for *cry1*, *cry2* and *vip3A*)	+	+	-	+
17.	4Q7 (Acrystalliferous *Bt* strain)	-	-	-	-
18.	4AT0 (Reference strain for *cry9*)	-	-	+	-

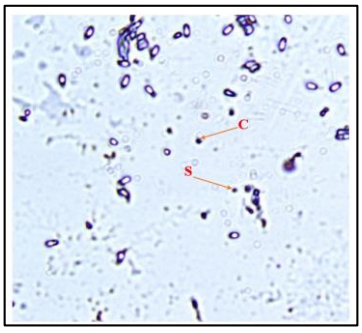

Fig. 1. Spore crystal inclusions of *Bt* isolates observed under a bright-field microscope (100 X magnification). C- Cuboidal; S- Spherical

Conclusion

Characterization and detection of insecticidal *cry* or *vip* gene (s) in the *Bt* isolates will be a base study and helpful for identifying the potential *Bt* isolate and in turn which can be used for the preparation of *Bt* biopesticide or isolation of insecticidal gene (s) for the development of insect-resistant transgenic plants.

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