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Characterization of Indigenous *Bacillus thuringiensis* Isolates and Toxicity Analysis Against Brinjal Shoot and Fruit Borer, *Leucinodes orbonalis* (Guenee)

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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

The toxins derived from *Bacillus thuringiensis* (*Bt*) exhibit significant promise in managing specific orders of harmful insects without the drawbacks in using of chemical insecticides. This study examines 20 indigenous *Bt* isolates, with a focus on their colony and crystal morphology, gene

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content, protein profiles, and toxicity against the larvae of *Leucinodes orbonalis*. The results revealed that all colonies were creamy white in colour with fried egg type appearance, flat surface, and undulated margin with spherical shaped crystals. PCR screening detected the existence of *cry1*, *cry2*, and *vip3A* genes with varying gene combinations among the isolates. Protein profiles exhibited the presence of multiple proteins with molecular weights ranging from 20 to 135 kDa, particularly ~135 and ~65 kDa represents Cry1 and Cry2 protein. In vitro bioassays against *L. orbonalis* revealed that four isolates T193, T339, T374, and T380 having substantial toxicity (93.33 to 100% mortality), suggesting the potential for their utilization in sustainable pest management strategies.

Keywords: Bacillus thuringiensis; insecticidal toxins; indigenous isolates; Leucinodes orbonalis; In vitro bioassay.

1. INTRODUCTION

Farmers across the world mostly depend on pesticides for pest management, but chemical control agents pose serious risks, including insect resistance, rise of secondary pest outbreak, harm to non-target organisms, environmental deterioration, and residues on crops and environment. Hence, there is a critical need for exploration of an eco-friendly pesticides [1,2, and 3]. Biological control offers a potential alternate solution by utilizing microorganisms to manage pest species [4,5]. *Bacillus* spp. stands out among the microorganisms employed for biological control, particularly in integrated pest management in agriculture, particularly, *Bacillus thuringiensis* based formulations constitute a dominant share, comprising up to 90% of the market [6,7,8] among bio pesticides.

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium found in diverse ecosystems, including soil, water, dead insect, and various plant ecosystems like phylloplanes and rhizosphere [9,10]. It has been recognized for its ability to produce crystalline inclusions containing insecticidal proteins called δ-endotoxin. *Bt* strains produce crystal (Cry) and cytolytic (Cyt) toxins during sporulation phase [11,12]. Furthermore, at vegetative phase, *Bt* isolates have the capability to generate additional insecticidal proteins, like vegetative insecticidal proteins (Vip) [10,13,14,15] and secreted insecticidal proteins (Sip) [16,17]. These cry proteins are effective in managing a diverse array of insect orders viz Lepidoptera, Coleoptera, Diptera, -Hymenoptera, Homoptera, Orthoptera and also exhibit toxicity towards nematodes [12,18,19,20,21,22]. These cry toxins can be categorized based on amino acid homology, and the proteins with the same primary rank in phylogenetic tree, typically exhibit toxicity to specific insect orders [23]. Over 800 *Cry* genes are categorized into 75 families (from Cry1 to Cry75), while 40 *Cyt* genes are grouped into three families (Cyt1, Cyt2, and Cyt3). Additionally, 146 *Vip* genes are classified under four families (Vip1 to Vip4) [24]. While *Bt* proves to be an effective alternative to synthetic insecticides for controlling important insect pests, its prolonged use has the drawback of developing resistance in target insect populations in both laboratory and field studies [25,26]. In order to combat these challenges, in the current study, toxicity of 20 indigenous *Bt* isolates were evaluated against *Leucinodes orbonalis* under laboratory conditions.

2. MATERIALS AND METHODS

2.1 Insect Culture

Laboratory culture of *L. orbonalis* was initiated from the damaged field collected brinjal fruits. The mass culturing of Brinjal Shoot and Fruit Borer (BSFB) was done by following the method outlined by Visnupriya and Muthukrishnan [27] with minor modification. These collected fruits were placed in a tray filled with double autoclaved river sand and were maintained under controlled environmental conditions, including temperature (25 ± 1 °C), humidity (75 ± 5%), and a photoperiod of 16: 8 hours (L:D). The larva pupates in the soil and emerged adults were provided a 10% sugar solution enriched with vitamin E and laid eggs in the black cloth. Newly hatched larvae were transferred to potato tubers, containing 4 – 5 longitudinal slits and allowed to grow and pupate in sand. After being reared for two generations under laboratory condition, a stable insect population was utilized for the bioassay studies.

2.2 *Bt* Isolates

Twenty native *Bt* isolates, Standard strain HD1 (*Btk*) and a-crystalliferous strain 4Q7 were

obtained from Bt Laboratory, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, India. These bacterial cultures were revived from glycerol stock and maintained in T3 medium (For a liter of media: 2 g of tryptose, 3 g of tryptone, 1.5 g yeast extract, 6.9 g sodium dihydrogen phosphate, 8.9 g disodium hydrogen phosphate, 100 µl of manganese chloride (0.05 g in 1 ml of water), 20 g Agar, pH – 6.8 to 7.0).

2.3 Colony and crystal morphology of *Bt* Strains

Colony characteristics like colour, type, elevation and margin of bacterial colonies were studied by visually observing a single isolated colony. To access the morphology, spore crystal inclusions were heat fixed on glass slides, stained with 0.133% Coomassie Brilliant Blue stain and examined under a bright field microscope at 100X magnification (Euromex iScope).

2.4 PCR Screening for Cry and Vip Genes

The extraction of genomic DNA from *Bt* strains was done, following the protocol of Kalman et al. [28]. The DNA concentration was measured using a Nano-Drop Spectrometer (Genova Nano, Jenway) and 0.8% agarose gel electrophoresis was employed to assess the quality of the DNA. PCR screening was done to confirm the presence of lepidopteran toxic genes, namely *cry1*, *cry2*, *cry9*, and *vip3A.* For carrying out PCR analysis, each reaction mixture consisted of 1 µl (50 ng) of template DNA, 1 µl of each primer (10 pmol), and 10 µl of 2X PCR Master Mix (Smartprime), comprising dNTPs, Taq polymerase, and 7 µl of sterile distilled water was used. The PCR temperature profile for each gene was maintained as specified in Table 1. Subsequently, the PCR products were separated in an agarose gel with EtBr (Ethidium bromide) staining and documented using a gel documentation system (Bio-Print imaging device, Vilber, France).

Table 1. Details of primers used and its temperature profile for PCR screening *Bt* isolates

S. No	Gene	Primer sequence	Product size	Temperature profile	Reference
1	*cry1*	FP: 5'-CATGATTCATGCGGCAGATAAAC-3’ RP: 5'-TTGTGACACTTCTGCTTCCCATT-3'	~ 277 bp	94 °C for 2 min 94 °C for 40 s 62 °C for 40 s 72 °C for 1 min 72 °C for 7 min 30 cycles	[29]
2	*cry2*	FP: 5'-GTTATTCTTAATGCAGATGAATGGG-3' RP: 5'-CGGATAAAATAATCTGGGAAATAGT-3'	~ 700 bp	94 °C for 2 min 94 °C for 40 s 60 °C for 40 s 72 °C for 40 s 72 °C for 10 min 30 cycles	
3	*cry9*	FP:5'-CGGTGTTACTATTAGCGAGGGCGG-3' RP: 5'-GTTGAGCCGCTTCACAGCAATCC-3'	~ 345 bp	59 °C for 40 s 61.5 °C for 40 s 72 °C for 1 min 72°C for 7 min 72°C for 1 min 25 cycles	
4	*vip3A*	FP: 5'-CCTCTATGTTGAGTGATGTA-3' RP: 5'-CTATACTCCGCTTCACTTGA-3	~ 1.0 Kb	94 °C for 5 min 94 °C for 1 min 55 °C for 1 min 72 °C for 40 s 72 °C for 10 min 35 cycles	[30]

2.5 Spore-crystal Mixture Isolation and Protein Profiling of *Bt* Isolates

Spore crystal mixture of *Bt* isolates and standard strains were obtained using standard procedure [31]. From each *Bt* culture, a single colony was carefully picked using a sterile loop and introduced into test tubes containing 5 ml of T3 broth. Subsequently, these cultures underwent an overnight incubation at 30 °C with continuous shaking at 200 rpm (Orbitek, Scigenics Biotech). Following this, a 1% inoculum (250 µl) from the overnight cultures was transferred into T3 broth (25 ml), and then incubated for 48 hours at 30 °C and 200 rpm. After observing 90% of the cells lysis at 48-hour period, the culture was subjected to centrifugation at 7500 rpm for 15 minutes (at 4°C). The resulting pellet was then resuspended in 500 µl of Sterile Water with 10 µl of 1mM phenyl methyl sulfonyl fluoride (PMSF) and stored at -20°C for subsequent use. Protein profiling of *Bt* isolates was done by using SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), involving 10% separating gel and 4% stacking gel [32].

2.6 Bioassay

With Bradford assay [33], to quantify the protein concentration in spore crystal mixture was quantified using Bovine Serum Albumin (BSA) as a standard. The insect bioassay was conducted against neonates of *L. orbonalis* through surface coating method at 25 µg/ml concentration [34] using potato. Ten first-instar larvae (pre-starved for 8 hours) were released onto the potato without any physical damage. The standard strain HD1 and water served as a positive and negative controls respectively. Each treatment was replicated thrice and observation on larval mortality were recorded up to 72hrs using a stereo zoom microscope (Labomed stereoscopic microscope CSM2, California, United States) and expressed in percentage.

2.7 Statistical Analysis

The laboratory experiment followed a completely randomized design (CRD). Using Abbott's formula [35], larval mortality was corrected for control mortality, and one-way analysis of variance (ANOVA) was done using R software Version 4.3.1 [36]. Subsequently, significant difference between means were identified through Tukey HSD test.

3. RESULTS AND DISCUSSION

3.1 Colony and Crystal Morphology of *Bt* Strains

The 20 screened *Bt* isolates were uniformly creamy white in color. Among them, 17 isolates exhibited fried egg type colony and 3 isolates showed mucoid colonies. In terms of colony shape and margin, 12 isolates showed circular colony with complete margin, whereas 8 isolates had irregular colony with undulated margin. Colony elevation of 15 isolates were flat while 5 isolates exhibited raised colonies (Table 2). Different types of crystal inclusions were observed among the isolates viz., spherical (10), bipyramidal (7), cuboidal (6), and rectangular (2). These findings were in accordance with the results of Navya et al. [34]. Rashki et al. [37] reported varying crystal shapes *viz.,* bipyramidal, spherical, cubical, irregular, and elliptical, of which spherical shape (73.33%) was the most abundant among the *Bt* isolates of Iran. According to Sathyan et al. [38], Scanning Electron Microscopy revealed the presence of bipyramidal, spherical, and cubic crystals in the spore suspension of T405.

Table 2. Colony morphology of *Bt* isolates

Colour	Type	Elevation	Margin	Shape	Bt isolates	Occurrence	
						Nos.	%
Creamy white	Fried egg	Flat	Complete	Circular	T52, T194, T195	3	15.0
				Irregular	T46, T380	2	10.0
			Undulated	Circular	T193, T197, T374	3	15.0
				Irregular	T100, T159, T196, T338, T339	5	25.0
		Raised	Complete	Circular	T51, T198, T199	3	15.0
				Irregular	T54	1	5.0
	Mucoid	Flat	Complete	Circular	T45, T53	2	10.0
		Raised	Complete	Circular	T158	1	5.0

Table 3. Distribution of *Cry* and *Vip* genes among indigenous *Bt* isolates

Bt isolates	Genes			
	cry1	cry2	cry9	vip3A
T45	-	-	-	-
T46	-	-	-	-
T51	-	-	-	-
T52	-	-	-	-
T53	-	-	-	-
T54	-	-	-	-
T100	-	-	-	-
T158	-	-	-	-
T159	-	-	-	-
T193	+	-	-	-
T194	+	-	-	-
T195	+	-	-	-
T196	+	-	-	-
T197	+	-	-	-
T198	-	-	-	-
T199	-	-	-	-
T338	-	-	-	-
T339	+	+	-	-
T374	+	+	-	+
T380	+	+	-	+
HD1	+	+	-	+
4AT1	-	-	+	-
4Q7	-	-	-	-

HD1 – positive standard strain for cry1, cry2 and vip3A; 4AT1 – standard strain for cry9 only; 4Q7 – negative strain for all genes. (+) = presence and (-) = absence of respective genes

3.2 PCR Screening of *Bt* Isolates

Examination of 20 *Bt* isolates indicated that five isolates possessed only *cry1* gene, three isolates harbored both *cry1* and *cry2* genes, and two isolates possessed *cry1*, *cry2*, and *vip3A* genes. No amplification was observed for *cry9* gene in any of the isolates. (Table 3). The coexistence of *cry* and *vip* genes in *Bt* isolates was formerly reported by Navya et al. [34] and Gothandaraman et al. [39]. Lone et al. [40] confirmed the existence of both *cry1* and *cry2* gene in 12 out of 44 isolates screened. Karuppaiyan et al. [41] and Maheesha et al. [42] reported the occurrence of *cry1A (cry1Ab, cry1Ac), cry2 (cry2Aa, cry2Ab)* and *vip3A* genes in the native *Bt* isolates using PCR analysis. *Cry1* was the most common gene among the 20 isolates screened followed by *cry2*, *vip3A* genes. Previously, *cry1* type genes were reported as predominant gene, present in indigenous *Bt* strains [43,44,45].

3.3 Sodium Dodecyl Sulphate–polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

The insecticidal crystal proteins of the *Bt* isolates varied in their molecular weight between 20 and 135 kDa (Table 4). Eight isolates showed protein bands of ~ 135 kDa size (Cry1) and three isolates with protein size of ~ 65 kDa (Cry2), respectively. Vip3 protein of size ~88 kDa was observed in two isolates (Fig. 1). Protein profiles were comprising of varying no. of bands: a single band in 1 isolate, two bands in 2 isolates, and more than 2 bands in 16 isolates, while one isolate did not exhibit any distinct band. Hassan et al. [26] reported multiple protein bands of 20 to 130 kDa size, among *Bt* strains. Of the 70 *Bt* strains that were screened through SDS-PAGE analysis, Ramalakshmi and Udayasuriyan [30] found that 17 strains (24.2%) showed two prominent protein bands, which corresponded to Cry1 and Cry2 protein, with molecular weights ~ 135 and 65 kDa. Sujayanand et al. [46] reported protein bands of various sizes viz., 175, 135, 65, 51, 35, and 21 kDa; including 135 and 65 kDa size bands of Cry1 and Cry2 protein. Similarly Kaviyapriya et al. [47], Karuppaiyan et al. [41], Maheesha et al. [42], and Reyaz et al. [48] reported the Cry1 and Cry2 proteins of 135 and 65 kDa size. Lone et al. [49] revealed that Vip3A protein produce bands at approximately 88 kDa in size.

3.4 Toxicity Analysis against *L. orbonalis*

The toxicity results revealed that two isolates (i.e.) T374 and T380 exhibited 100% mortality as similar as positive standard strain HD1 and two isolates, T193 and T339 showed 93.33% mortality. *Bt* isolates T195, T196, and T197 showed mortality ranging from 75 to 80%. Five *Bt* isolates T45, T46, T51, T53, and T159 showed least toxicity (≤ 10%) and four isolates viz., T52, T54, T100, and T158 recorded no mortality (Table 5). Sunitha and Kalia [3] reported the insecticidal activity of twenty-six indigenous *Bacillus* spp. With mortality rates ranging from 52% to 100% against *L. orbonalis*. Satpathy et al. [50] reported bioassay data using *cry1Ac* incorporated artificial diet with 94.77% mortality in *L. orbonalis*. Similarly, Ramalakshmi et al. [44] recorded 16.7 – 100% mortality among 17 isolates of *Bt* against *Helicoverpa armigera*. Out of 56 *Bt* isolates screened by lone et al. [40], four isolates (JK12, JK22, JK48, and JK72) produced 96 – 100% mortality in *H. armigera*. Reyaz et al. [51] found that *Bt* isolate T414 harboring both *cry1* and *cry2* genes, showed 100% mortality in *Pectinophora gossypiella* under laboratory condition. Gemmeda et al. [52] screened ten native Bt isolates against 3rd instar larvae of H. armigera, resulted in high mortality of 75.83%.

Table 4. Various protein bands produced by *Bt* isolates

Protein size	Occurrence (n= 20)	
	Nos.	%
~ 135 kDa	8	40.0
~ 100 kDa	3	15.0
~ 91 kDa	2	10.0
~ 88 kDa	4	20.0
~ 80 kDa	1	5.0
~ 71 kDa	2	10.0
~ 65 kDa	7	35.0
~ 54 kDa	7	35.0
~ 52 kDa	5	25.0
~ 50 kDa	10	50.0
~ 43 kDa	3	15.0
~ 40 kDa	4	20.0
~ 33 kDa	1	5.0
~ 30 kDa	6	30.0
~ 27 kDa	13	65.0
~ 25 kDa	2	10.0
~ 20 kDa	1	5.0

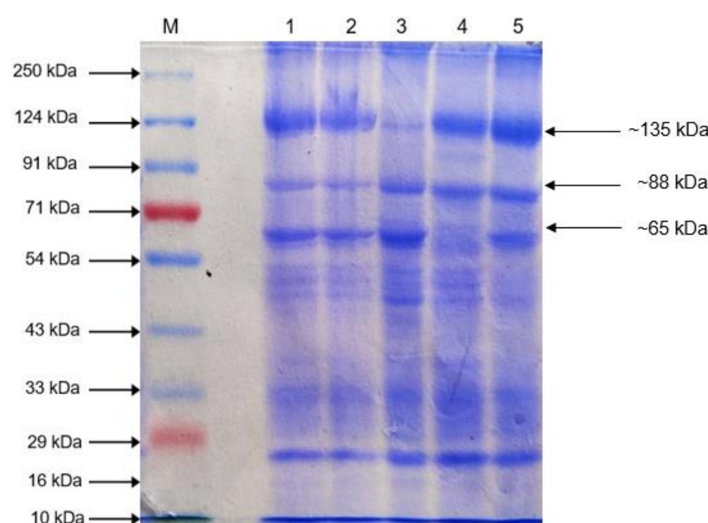

Fig. 1. SDS-PAGE profile of *Bt* isolates

Lane M – Protein marker, Lane 1 – T380, Lane 2 – T374, Lane 3 – T339, Lane 4 – T193, Lane 5 – HD1

Table 5. Percent mortality of *L. orbonalis* caused by indigenous *Bt* isolates (@ 25 μg/ml)

S. No.	*Bt* isolates	% Mortality at 72 HAT
1	T45	10.0 (18.43)^{fg}
2	T46	3.33 (10.51)^{g}
3	T51	6.66 (14.96)^{g}
4	T52	0.00 (0.91)^{g}
5	T53	10.0 (18.43)^{fg}
6	T54	0.00 (0.91)^{g}
7	T100	0.00 (0.91)^{g}
8	T158	0.00 (0.91)^{g}
9	T159	3.33 (10.51)^{g}
10	T193	93.33 (75.03)^{abc}
11	T194	66.66 (54.73)^{cd}
12	T195	80.0 (63.43)^{bc}
13	T196	80.0 (63.43)^{bc}
14	T197	76.66 (61.11)^{bc}
15	T198	33.33 (35.26)^{def}
16	T199	30.0 (33.21)^{ef}
17	T338	40.0 (39.23)^{de}
18	T339	93.33 (75.03)^{abc}
19	T374	100 (89.09)^{a}
20	T380	100 (89.09)^{a}
21	HD1	100 (89.09)^{a}
22	Control	0.00 (0.91)^{g}

**HAT – Hours After Treatment*

Figures in parentheses are arcsine transformed values of percentages. Values followed by the same letters in a column are not significantly different by the Tukey Honestly Significant Difference (HSD) test with α = 0.05 (F = 79.04; df = 21, 44; P < 0.001)

4. CONCLUSION

Four out of 20 *Bt* isolates exhibited higher toxicity (93.33 – 100%) against *L. orbonalis*. Identification of toxic genes cry1 and cry2 in these *Bt* isolates can be utilized in development of transgenic plants. These effective isolates can also be used for formulation studies. Overall, these findings underscore the multifaceted nature of *Bt* isolates and their promising role as an effective bio pesticides for insect control.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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