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Molecular characterization and toxicity evaluation of indigenous *Bacillus thuringiensis* isolates against key lepidopteran insect pests

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Abstract

Background: The indiscriminate use of synthetic insecticides is not affordable and poses collateral damage to humans, non-target organisms, and environmental health. *Bacillus thuringiensis* (*Bt)* is a cosmopolitan, antagonistic soil bacterium employed as an alternative to chemical insecticides in pest management. In this study, genetic diversity and insecticidal activity of 50 indigenous *Bt* isolates were investigated in order to find the effective *Bt* isolates with a broad spectrum of insecticidal activity against lepidopteran insects.

Results: Most of the *Bt* isolates investigated appeared as creamy white in colour and fried egg or mucoid-type colonies having a flat or raised elevation with entire or undulated margins. Out of 50 isolates investigated, crystal shapes varied from cuboidal (40.00%), spherical (36.00%), bipyramidal (30.00%), rectangular (10.00%), and minute crystal attached to the spores (28.00%). SDS-PAGE analysis revealed that the molecular weight of the crystal proteins of *Bt* isolates ranged from ~ 30 to ~ 200 kDa in size. Among the *Bt* isolates screened, ~ 135 kDa size representing Cry1 protein was observed in 12 isolates (24.00%) and 65 kDa representing Cry2 was observed in 14 isolates (28.00%). PCR analysis was performed for *cry1, cry2, cry3, cry4, cry9, vip1, vip2,* and *vip3* genes, which revealed the presence of *cry1* gene alone in 5 isolates, and *cry2* alone in 7 isolates, whereas 7 isolates were positive for *cry1, cry2,* and *vip3* genes together. None of the *Bt* isolates showed the presence of *cry3, cry4, cry9, vip1,* and *vip2* genes. Toxicity of *Bt* isolates was tested against four species of lepidopteran larvae, viz. *Plutella xylostella* Linnaeus, *Helicoverpa armigera* Hubner, *Spodoptera litura* Fabricius, and *S. frugiperda* Smith. Among them, 6 isolates (T29, T30, T31, T357, T381, and T388) produced 100% larval mortality against all four species of insects.

Conclusion: The present study showed the diversity of *Bt* isolates and confirmed the significance of relentless exploration of *Bt* isolates for novel genes. Further investigations need to be carried out to disclose the hidden potential of these toxic isolates.

Keywords: *Bacillus thuringiensis*, Diversity, Crystals, Cry protein, Toxin, Lepidopterans

Background

Bacillus *thuringiensis* (*Bt*) is a gram-positive, spore-forming entomopathogenic bacterium that produces different proteins toxic to many insects, nematodes, mites, and also protozoans (Santos et al. 2022). It is diversified all over the world usually found in soil, leaf surfaces, grain dust, dead insects, and aquatic environment (Gupta et al. 2021). The insecticidal proteins were produced during the

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stationary phase as crystal inclusions (crystal (Cry) and cytolytic (Cyt) proteins) or secreted during the vegetative phase (vegetative insecticidal proteins (Vip)) of bacterial growth (Adang et al. 2014). Among these, Cry and Cyt proteins are most explored as biopesticides for their toxicity against various agricultural pests and vectors of human diseases. Crystalline toxins or δ-endotoxins are considered as the main factor conferring insecticidal properties to *Bt* (Bouslama et al. 2020). Commercialized *Bt* delta toxins have been used as pesticides over decades as they have constitutive blocks of amino acids and different specificities against different orders of insects (Van Frankenhuyzen 2009). δ-endotoxins of *Bt* attack the insects' membrane pores and form channels (Melo et al. 2016). The three-domain proteins have various complementary aspects of insect toxification. The first domain is responsible for the formation of pores; the second domain is specific in binding to the receptors in the epithelial cells of the insects' midgut; the third domain is functioning in stabilizing the bond between toxin and receptor that results in osmatic discrepancy and finally death of insect (Melo et al. 2016). Numerous *Bt* strains that showed toxicity towards a wide range of insect pests, viz. lepidopterans, dipterans, coleopterans, homopterans, hymenoptera, mallophaga, and nematodes, have been isolated from different sources and used for developing biopesticides (Abo-Bakr et al. 2020). The crystal proteins, viz. Cry1, Cry2, Cry9, and Vip3A, were found to be toxic and specific to various lepidopteran insect pests. At present, about 400 *Bt*-based biopesticide formulations made up of insecticidal proteins and spores have been registered and commercially used in the market. These toxins are employed in pest management in the form of biopesticides or by expressing the toxin-encoded genes in commercial crops against target insect pests (George and Crickmore 2012).

Based on the amino acid sequence homology, the insecticidal proteins were classified into different groups and so far 80 holotypes of crystal proteins (Cry1–Cry 80), four holotypes of vegetative insecticidal proteins (Vip1–Vip4), and seven holotypes of cytolytic proteins (Cyt1–Cyt7) of *Bt* have been identified and reported (Crickmore et al. 2018). Cry toxins are highly selective and specific to their target insect, inoffensive to non-target pests, vertebrates, and humans, and expeditiously degrade in the environment. Therefore, *Bt* is an effective alternative to synthetic insecticides for the management of agriculturally important insect pests. However, long-term use of *Bt* toxins leads to the development of resistance to the toxins by the target insect pests (Hassan et al. 2021). As a result, exploring new strains of *Bt* with novel toxins will provide another way to mitigate these problems. With this background, the present investigation was carried out to characterize 50 indigenous *Bt* isolates to explore their diversity and toxicity against key lepidopteran insect pests.

Methods

Indigenous *Bt* isolates and growth conditions

A total of 50 indigenous *Bt* isolates were obtained from *Bt* laboratory stock, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India (Additional file 1: Table S1). The reference strains for a different group of Cry toxins originally received from Bacillus Genetic Stock Center (BGSC, Columbus, Ohio) were used as a positive check. The *Bt* cultures were revived from glycerol stocks on T3 agar medium (per litre: 3.0 g Tryptone, 2.0 g Tryptose, 1.5 g Yeast extract powder, 6.9 g NaH_2PO_4; 8.9 g Na_2HPO_4 and 0.005 g MnCl; 10.0 g agar (pH 6.8–7.0) and incubated at 30 °C for 14 h. In order to get single colonies, the overnight grew *Bt* cultures were subcultured on T3 agar medium by quadrant streak method and incubated at 30 °C for overnight. Purified single colonies were inoculated separately in test tubes containing 5 ml of T3 broth and incubated at 30 °C for 24 h with 200 rpm (Orbitek, Scigenics Biotech India Pvt Ltd., Chennai, India).

Bacterial colony and crystal morphology

The colony morphology of individual *Bt* isolates was examined visually. A loop full of *Bt* culture was inoculated in 5 ml of T3 broth and incubated at 30 °C for 48 h at 200 rpm. To check the spore-crystal inclusions in the *Bt* isolates, a loop full of culture smears was prepared in glass slides, heat-fixed, and stained with the 0.133% Coomassie Brilliant Blue stain (G250). Then, the stained-glass slides were washed gently with water, blot dried with tissue paper, and observed under a bright-field microscope for the existence of crystalline inclusion (Leica DM 1000LED, DFC295, Germany).

Preparation of spore-crystal mixtures from *Bt* isolates

A single colony of *Bt* cultures was inoculated into 5 ml of T3 broth and incubated at 30 °C overnight at 200 rpm. After the overnight incubation, 1% grown cultures were inoculated into a 250-ml conical flask containing 25 ml of T3 broth and incubated at 30 °C with shaking of 200 rpm for 48–60 h. The bacterial sporulation was observed under a bright-field microscope (Leica DM 1000LED, DFC295, Germany). When more than 90% of cells were lysed, the sporulated cultures were transferred into 50-ml falcon tubes and incubated at 4 °C for half-an-hour before harvest. The sporulated bacterial cultures were centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended

in 25 ml of ice-cold Tris–EDTA buffer (10 mM Tris; 1 mM EDTA and pH 8.0) containing 1 mM PMSF (phenyl methyl sulphonyl fluoride) and washed thrice with Tris–EDTA buffer and once with 25 ml of ice-cold 0.5 M NaCl solution containing 0.5 mM PMSF by centrifugation at 8000 rpm for 10 min at 4 °C (Eppendorf centrifuge 5810R, Germany). The final pellet was dissolved in nuclease-free water containing 1 mM PMSF and stored at − 20 °C (Ramalakshmi and Udayasuriyan, 2010).

Protein profiling of *Bt* isolates

Protein profiling was done using SDS-PAGE (Sodium dodecyl sulphate poly acrylamide gel electrophoresis) by the method of Laemmli (1970) using 10% separating and 4% stacking gel. The spore crystal mixture samples were prepared by mixing with 4× loading dye (0.25 M Tris HCl pH 6.8; 8% SDS, 40% glycerol, 0.5% bromophenol blue) in a ratio of 4:1. Then, the samples were incubated in boiling water for 2 min before loading. The molecular weight of the protein was estimated by using three colour pre-stained protein marker (PG PMT 2922, Puregene, Genetix Biotech Asia Pvt Ltd.,) covering a broad range of molecular weights from 10 to 250 kDa.

Bacterial genomic DNA isolation and polymerase chain reaction

Purified single colonies of *Bt* isolates were used for genomic DNA isolation by following Sambrook and Russell's (2001) method. Extracted genomic DNA was used as a template DNA (30–50 ng) for the amplification of *cry1, cry2, cry3, cry4, cry9, vip1, vip2,* and *vip3* genes with their specific primers (Additional file 1: Table S2). A total reaction volume of 20 µl consists of 1 µl of template DNA, 1 µM of each primer, 10 µl of 2X PCR master mix (SmartPrime) consisting of dNTPs, Taq polymerase, and 7 µl of nuclease-free water. PCR analysis was performed in Mastercycler Nexus GX2 (Eppendorf, Germany), and the PCR products were resolved in agarose gel electrophoresis with ethidium bromide staining. The products were visualized under a UV trans-illuminator for the expected size of amplification of different *cry* and *vip* genes (Additional file 1: Table S2).

Insect cultures

The insect cultures of *Plutella xylostella* (pupae), *Spodoptera litura* (eggs), *S. frugiperda* (eggs), and *Helicoverpa armigera* (eggs) were obtained from NBAIR (National Bureau of Agricultural Insect Resources, Bangalore, India) and established in insect bioassay laboratory at Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore, India. The newly emerged adult *P. xylostella* was supplemented with a 10% sugar solution along with Vitamin E, and two leaf-stage mustard seedlings were provided as an oviposition substrate. Newly hatched neonate larvae were transferred into Cauliflower leaf (maintained under controlled conditions) and were maintained at the laboratory with fresh cauliflower leaves as and when required. The cultures of *S. litura, S. frugiperda* and *H. armigera* larvae were reared on an artificial diet. Pupae were collected and allowed for adults' emergence in an insect-rearing cage (2.5 × 2.5 Ft). Newly emerged adult moths were supplemented with 10% sugar solution along with Vitamin E. Young maize seedlings for *S. frugiperda* and *H. armigera*, and *Nerium oleander* shoots for *S. litura* were placed inside the cage as an oviposition substrate. Eggs were collected and maintained in Petri plates for hatching. Newly hatched neonates were transferred into an artificial diet by using a camel hairbrush and maintained.

Toxicity of *Bt* isolates against lepidopteran insects

The concentration of spore-crystal mixtures was estimated by Broadford's reagent method (Bradford 1976). Estimated spore-crystal mixtures were equalized to a concentration of 20 µg/ml and used for in vitro insect bioassay. Detached leaf bit bioassay was carried out with insect-specific host plants. Cauliflower leaves were used for *P. xylostella* and *S. litura*, while maize leaves were used for *S. frugiperda* and *H. armigera*. Circular leaf bits of cauliflower (2 cm dia) were prepared and coated with the spore-crystal mixture on both sides (10 µl/side) of the leaf disc and air-dried. Similarly, 2 cm leaf bits of maize leaves were treated with spore-crystal mixtures (10 µl/side). Treated leaf bits were placed into a 3 cm dia plastic container containing moist filter paper and pre-starved neonate larvae were released. Ten larvae were used per treatment and replicated thrice. The larval mortality was observed up to 72 h after treatment and expressed in percentage. The entire in vitro insect bioassay experiments were conducted under a controlled environment with 25 ± 1 °C and 65 ± 2% RH.

Statistical analysis

The laboratory experiments were performed in a completely randomized design (CRD). The larval mortality data were computed using Abbott's formula (Abbott 1925), and subjected to one-way variance (ANOVA) in AGRES statistical software version 7.01, and significant differences between means were determined by Duncan's multiple range test (DMRT) at $p = 0.05$.

Results

Bacterial colony and crystal morphology

All the bacterial colonies screened were creamy white in colour. Colony type was observed as fried egg type with raised elevation in 12 *Bt* isolates and flat elevation

Table 1 Colony morphology of indigenous *Bt* isolates

Sl. No	Colony parameter					*Bt* isolate	% Occurrence
	Colour	Shape	Colony type	Elevation	Margin		
1	Creamy white	Circular	Fried egg	Raised	Entire	T7, 10, T28, T59, T62, T73, T220	14.00
2	Creamy white	irregular	Fried egg	Raised	Undulate	T9, T381, T55, T76, T77	10.00
3	Creamy white	Circular	Fried egg	Flat	Entire	T17, T58, T60, T63, T69, T79, T81, T83, T250, T385, T386, T387, T388, T389	28.00
4	Creamy white	Circular	Fried egg	Flat	Undulate	T29, T30, T31, T353, T355, T357	12.00
5	Creamy white	Irregular	Fried egg	Flat	Entire	T67, T78	4.00
6	Creamy white	Irregular	Fried egg	Flat	Undulate	T82, T390, T392, T393, T395, T396, T397, T398, T399	18.00
7	Creamy white	Circular	Mucoid	Flat	Entire	T47, T48, T49, T56, T61, T71	12.00
8	Creamy white	Circular	Mucoid	Raised only in margins	Entire	T74	2.00

Table 2 Crystal shapes of *Bt* isolates

Crystal shape	Number of *Bt* isolates (out of 50)	% of occurrence
Bipyramidal	15	30.00
Cuboidal	20	40.00
Spherical	18	36.00
Crystals attached to spores	14	28.00
Rectangular	5	10.00

in 31 isolates, whereas mucoid-type colonies were found in six isolates with flat elevation and one isolate with raised elevation only in margins. The colony shape appeared circular in 34 *Bt* isolates and irregular in 16 isolates. The colony margin of 20 isolates was undulated and the remaining were entire (Table 1). Spore-crystal inclusions were observed under a bright-field microscope (100×) (Leica DM 1000LED, DFC295, Germany). Different types of crystal protein morphology were observed in 50 isolates varying from cuboidal (40.00%), spherical (36.00%), bipyramidal (30.00%), rectangular (10.00%), and minute crystal attached to the spores (28.00%) (Table 2; Fig. 1).

Protein profiling of *Bt* isolates

SDS-PAGE analysis revealed that the molecular weight of the parasporal crystal proteins of *Bt* isolates ranged from 30 to > 200 kDa in size. Among the *Bt* isolates screened, 135 kDa size representing Cry1 protein was observed in 12 isolates (24.00%) and 65 kDa representing Cry2 was observed in 14 isolates (28.00%) (Table 3; Fig. 2). Protein banding patterns were distributed as one prominent band in 16 isolates, two in 16 isolates, and more than three bands were recorded in 11 isolates, whereas seven isolates did not show any discrete band (Additional file 1: Table S3).

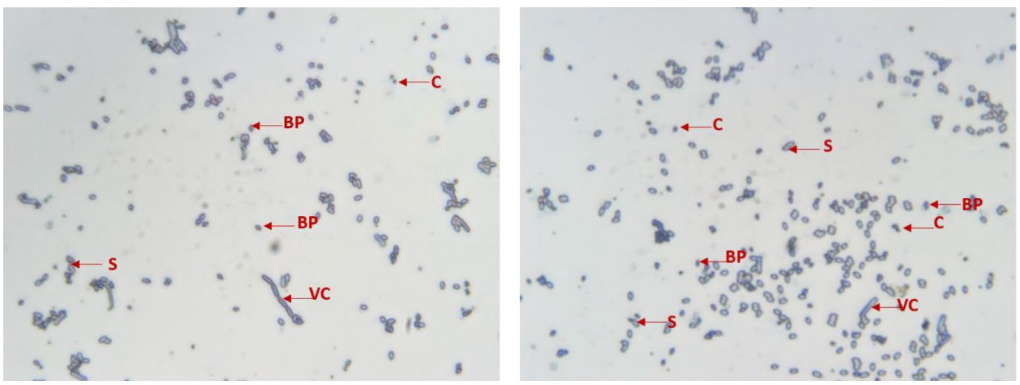


Fig. 1 Spore-crystal inclusions of *Bt* isolates observed under a bright-field microscope (100× magnification). VC—vegetative cells; S—spores; C—cuboidal; BP—bipyramidal

Table 3 Protein profile of indigenous *Bt* isolates from SDS-PAGE analysis

Protein size	*Bt* isolates	% occurrence
> 200 kDa	T55, T76 and T78	6
~ 145 kDa	T47, T48, T49, T73 and T79	10
~ 135 kDa	T17, T28, T29, T30, T31, T220, T250, T353, T355, T357, T381 and T388	24
~ 120 kDa	T79	2
~ 100 kDa	T73, T79, T392 and T393	8
~ 90 kDa	T71 and T74	4
~ 80 kDa	T67, T69, T74 and T81	8
~ 70 kDa	T67, T69, T71, T73, T74, T79 and T83	14
~ 65 kDa	T7, T10, T29, T30, T31, T81, T82, T355, T357, T381, T386, T387, T388 and T389	28
~ 55 kDa	T9, T55, T62, T67, T69, T73, T74 and T76	16
~ 45 kDa	T55, T56, T58, T59, T60, T61, T62, T63, T67, T69, T71, T73, T74, T76, T79 and T83	32
~ 30 kDa	T7, T9, T10, T55, T56, T58, T59, T60, T67, T71, T73, T74, T76, T77, T78, T79, T81, T82 and T83	38

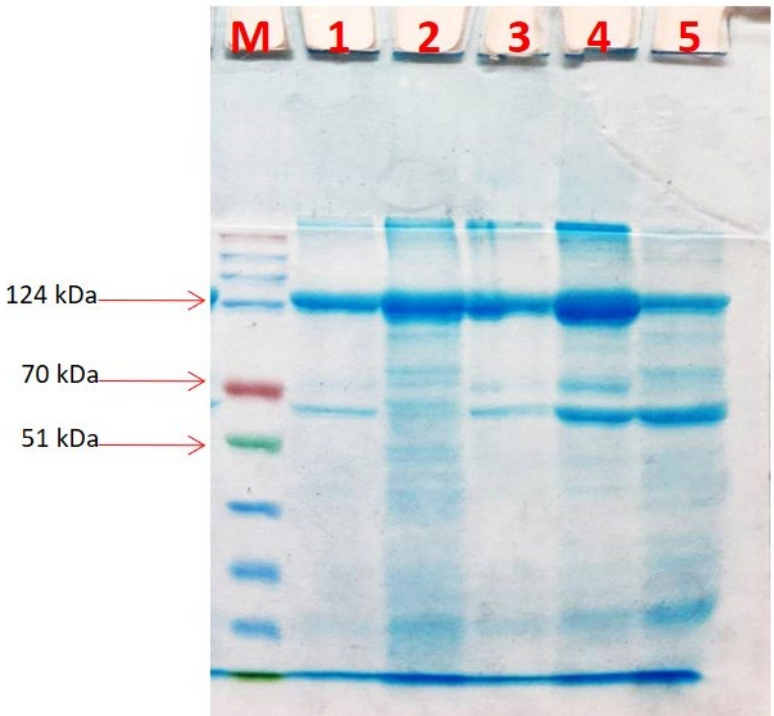


Fig. 2 Protein profile of *Bacillus thuringiensis* isolates. Lane M—Protein marker, Lane 1—HD-1, Lane 2—T28, Lane 3—T30, Lane 4—T357, Lane 5—T381

Insecticidal gene profile of *Bt* isolates

PCR analysis was performed for *cry1, cry2, cry3, cry4, cry9, vip1, vip2,* and *vip3* genes in PCR mastercycler (Eppendorf, Nexus GX2, Germany). Out of 50 isolates screened, five isolates were positive for *cry1* gene alone and seven for *cry2* gene alone, whereas seven isolates were positive for *cry1, cry2,* and *vip3* genes together (Tables 4; Additional file 1: Table S3 and Fig. 3). None of the *Bt* isolates showed the presence of *cry3, cry4, cry9, vip1,* and *vip2* genes.

Toxicity of *Bt* isolates against lepidopteran pests

Out of 50 *Bt* isolates tested against *P. xylostella,* 14 isolates produced 100% larval mortality and three isolates produced more than 90% mortality (Table 5). Bioassay against *H. armigera* revealed that 12 isolates were able

Table 4 Insecticidal gene profile of indigenous *Bt* isolates from PCR screening

Insecticidal gene profile	*Bt* isolates	% occurrence
cry1	T17, T28, T29, T30, T31, T220, T250, T353, T355, T357, T381, and T388	24
cry2	T7, T10, T29, T30, T31, T81, T82, T355, T357, T381, T386, T387, T388 and T389	28
cry3	–	–
cry4	–	–
cry9	–	–
vip1	–	–
vip2	–	–
vip3	T29, T30, T31, T355, T357, T381 and T388	14
cry1 + *cry2*	T29, T30, T31, T355, T357, T381 and T388	14
cry1 + *cry2* + *vip3*	T29, T30, T31, T355, T357, T381 and T388	14

to produce 100% mortality (Table 5). Similarly, the bioassay against *S. litura* and *S. frugiperda,* revealed that seven and six isolates were found to produce 100% mortality, respectively (Table 5). Out of 50 isolates screened, six isolates, viz. T29, T30, T31, T357, T381, and T388, produced 100% larval mortality against all the test insects (Figs. 4 and 5). One isolate (T393) produced no mortality in all four species, the remaining isolates produced larval mortality ranging from 3.33 to 96.67% in at least one species of insect. Out of 50 isolates tested, 14 isolates were able to cause 100% larval mortality in *P. xylostella,* followed by *H. armigera* (12 isolates), *S. litura* (7 isolates) and *S. frugiperda* (6 isolates) (Figs. 5 and 6).

Discussion

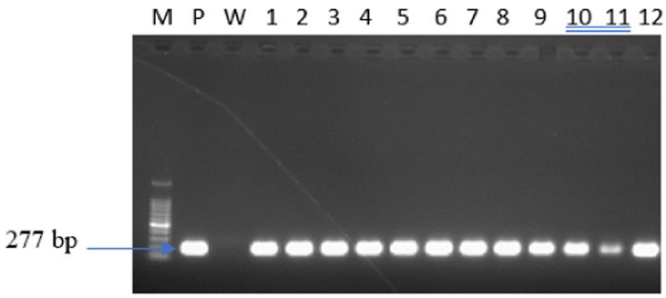
The diversified *Bacillus thuringiensis* (*Bt*) are found worldwide in different ecological habitats, viz. soil, sediment, stored products, insect cadaver, phylloplane, and aquatic environments (Baig et al. 2010). *Bt* producing insecticidal proteins are highly specific to the target insect pests (De Maagd et al. 2001). Hence, the characterization of *Bt* isolates from different geographical areas may lead to getting novel *Bt* proteins with a high level of insecticidal activity or broaden the insect spectrum.

The *Bt* isolates investigated appeared as creamy white in colour and fried egg or mucoid type colonies having flat or raised elevation with entire or undulated margins. Navya et al. (2021) observed creamy white to off-white colour colonies with fried egg appearance, irregular shape, flat and undulate margin in the *Bt* isolates screened.

The *Bt* bacterium was identified mainly based on the presence of parasporal crystalline inclusions. In the present study, out of the 50 isolates investigated, crystal shapes varied from cuboidal, spherical, bipyramidal, rectangular, and crystal attached to the spores. These findings are in accordance with reports by Ramalakshmi and Udayasuriyan (2010) wherein the *Bt* isolates with cuboidal (26.90%) and bipyramidal (21.00%) shapes were predominant when compared to other shapes, whereas these findings varied from the previous reports by Navya et al. (2021) from India, and Nair et al. (2018) from Qatar wherein more frequency of spherical crystals were observed. *Bt* isolates producing bipyramidal and cuboidal shapes of crystal were found to be highly toxic to lepidopteran insect pests (Boonmee et al. 2019). Variations observed in the morphology of the crystal shapes in the *Bt* isolates suggest the presence of diversity in *Bt* isolates in Tamil Nadu.

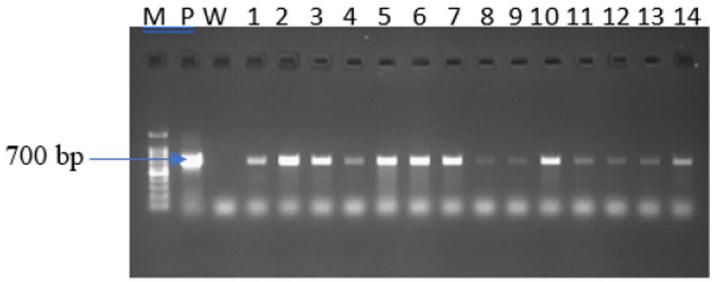
Analysis of crystal protein(s) profile could be useful to predict the presence of insecticidal genes. Among the *Bt* isolates screened, 135 kDa size representing Cry1 protein was observed in 12 isolates (24.00%) and 65 kDa representing Cry2 was observed in 14 isolates (28.00%). There are protein bands of different molecular weights between 30 and > 200 kDa in size observed which need to be characterized. Diversified electrophoretic patterns in Cry protein with molecular weights ranging from 20 to 160 kDa were reported earlier by Navya et al. (2021). In the present study, 16 isolates were found to produce one protein band (32.00%) and 16 isolates produce two protein bands (32.00%). Most of the isolates (28.00%) produced protein bands at ~ 65 kDa size representing Cry2 protein, followed by Cry1 protein (135 kDa) (Additional file 1: Table S3). Ramalakshmi and Udayasuriyan (2010) found that out of 70 isolates analysed, 17 isolates (24.20%) had two major protein bands with molecular weights in the range of ~ 135 and ~ 65 kDa. The diversity in the protein profile of *Bt* isolates indicates the possibility of diverse *cry* genes and corresponding insecticidal activities.

PCR analysis of 50 isolates indicates that five isolates were positive for *cry1* gene alone and seven for *cry2* gene alone, whereas seven isolates were positive for *cry1, cry2,* and *vip3* genes together. These findings were in



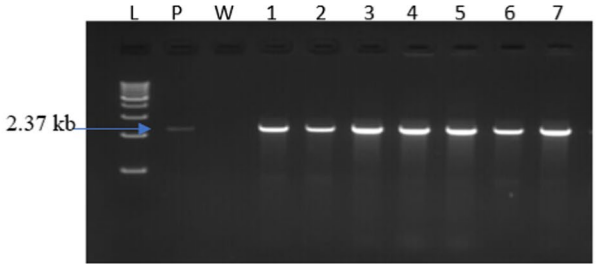
cry1

Lane M – 100 bp ladder, Lane P – positive control, Lane W – negative control, Lane 1 – T17; Lane 2 – T28; Lane 3 – T29; Lane 4 – T30; Lane 5 – T31; Lane 6 – T220; Lane 7 – T250; Lane 8 – T353; Lane 9 – T355; Lane 10 – T357; Lane 11 – T381; Lane 12 – T388



cry2

Lane M – 100 bp ladder, Lane P – positive control, Lane W – negative control, Lane 1 – T7; Lane 2 – T10; Lane 3 – T29; Lane 4 – T30; Lane 5 – T31; Lane 6 – T81; Lane 7 – T82; Lane 8 – T355; Lane 9 – T357; Lane 10 – T381; Lane 11 – T386; Lane 12 – T387; Lane 13 – T388; Lane 14 – T389



vip3

Lane L – 1 Kb ladder, Lane P – positive control, Lane W – negative control, Lane 1 – T29; Lane 2 – T30; Lane 3 – T31; Lane 4 – T355; Lane 5 – T357; Lane 6 – T381; Lane 7 – T388

Fig. 3 PCR amplification of *cry* and *vip* genes

accordance with the earlier report by Navya et al. (2021), confirming the presence of both *cry* and *vip* genes in combinations. Sahin et al. (2018) revealed that *vip3A1* positive isolates also had *cry1* and *cry2* genes, which are comparable to our findings.

Analysis of the insecticidal activity of spore crystal mixtures is an effective way to identify the spectrum of activity of indigenous *Bt* isolates (Sahin et al. 2018). In the present study, four lepidopteran insect pests, viz. *P. xylostella, H. armigera, S. litura,* and *S. frugiperda*, were selected to identify the effective *Bt* isolates. Out of 50 isolates screened, six isolates, viz. T29, T30, T31, T357, T381, and T388, produced 100% larval mortality against all four test insects. One isolate (T393) produced no mortality in all four species, the remaining isolates produced larval mortality ranging from 3.33 to 96.67% in at least

Table 5 Toxicity of indigenous *Bt* isolates against Lepidopteran pests

Sl. No	*Bt* isolates	Larval mortality (%) 72 h after treatment (20 µg/ml)			
		P. xylostella	*H. armigera*	*S. litura*	*S. frugiperda*
1	T7	96.67 (79.49)^{ab}	60.00 (50.77)^{e}	56.67 (48.83)^{ef}	70.00 (56.79)^{cd}
2	T9	0.00 (0.52)^{m}	0.00 (0.52)^{i}	0.00 (0.52)^{o}	13.33 (21.41)^{hi}
3	T10	100.00 (90.00)^{a}	83.33 (65.90)^{d}	53.33 (46.91)^{ef}	3.33 (10.51)^{kl}
4	T17	100.00 (90.00)^{a}	96.67 (79.49)^{b}	63.33 (52.73)^{de}	20.00 (26.56)^{gh}
5	T28	100.00 (90.00)^{a}	100.00 (90.00)^{a}	90.00 (71.56)^{b}	90.00 (71.56)^{b}
6	T29	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
7	T30	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
8	T31	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
9	T47	56.67 (48.83)^{d}	20.00 (26.56)^{f}	36.67 (37.27)^{hi}	13.33 (21.42)^{hi}
10	T48	13.33 (21.41)^{ghijk}	50.00 (45.00)^{e}	40.00 (39.23)^{gh}	0.00 (0.52)^{l}
11	T49	20.00 (26.56)^{ghi}	6.67 (14.97)^{gh}	36.67 (37.27)^{hi}	0.00 (0.52)^{l}
12	T55	0.00 (0.52)^{m}	16.67 (24.10)^{f}	16.67 (24.10)^{jkl}	23.33 (28.88)^{g}
13	T56	0.00 (0.52)^{m}	0.00 (0.52)^{i}	13.33 (21.41)^{klm}	6.67 (14.95)^{jk}
14	T58	10.00 (18.43)^{ijk}	0.00 (0.52)^{i}	10.00 (18.43)^{lm}	10.00 (18.43)^{ij}
15	T59	33.33 (35.26)^{ef}	0.00 (0.52)^{i}	13.33 (21.41)^{klm}	16.66 (24.09)^{ghi}
16	T60	13.33 (21.41)^{ghijk}	3.33 (10.51)^{h}	0.00 (0.52)^{o}	0.00 (0.52)^{l}
17	T61	26.66 (31.09)^{efg}	6.67 (14.97)^{gh}	3.33 (10.51)^{no}	13.33 (21.41)^{hi}
18	T62	26.66 (31.09)^{efg}	0.00 (0.52)^{i}	3.33 (10.51)^{no}	6.67 (14.96)^{jk}
19	T63	3.33 (10.51)^{lm}	6.67 (14.97)^{gh}	13.33 (21.41)^{klm}	10.00 (18.43)^{ij}
20	T67	20.00 (26.56)^{fghij}	10.00 (18.43)^{hi}	10.00 (18.43)^{lm}	6.67 (14.96)^{jk}
21	T69	13.33 (21.41)^{hijk}	0.00 (0.52)^{i}	0.00 (0.52)^{o}	0.00 (0.52)^{l}
22	T71	13.33 (21.41)^{ghijk}	10.00 (18.43)^{fg}	16.67 (24.10)^{klm}	16.66 (24.09)^{ghi}
23	T73	10.00 (18.43)^{ijk}	10.00 (18.43)^{fg}	0.00 (0.52)^{o}	3.33 (10.51)^{kl}
24	T74	10.00 (18.43)^{jkl}	0.00 (0.52)^{i}	3.33 (10.51)^{no}	0.00 (0.52)^{l}
25	T76	23.33 (28.88)^{fgh}	13.33 (21.41)^{fg}	3.33 (10.51)^{no}	0.00 (0.52)^{l}
26	T77	6.66 (14.96)^{kl}	3.33 (10.51)^{hi}	0.00 (0.52)^{o}	0.00 (0.52)^{l}
27	T78	30.00 (33.21)^{ef}	16.67 (24.10)^{f}	0.00 (0.52)^{o}	6.67 (14.96)^{jk}
28	T79	13.33 (21.41)^{ghijk}	13.33(21.41)^{fg}	26.67 (31.09)^{ij}	6.67 (14.96)^{jk}
29	T81	73.33 (58.91)^{c}	13.33 (21.41)^{fg}	10.00 (18.43)^{lm}	6.67 (14.96)^{jk}
30	T82	53.33 (46.91)^{d}	16.67 (24.10)^{f}	6.67 (14.96)^{mn}	0.00 (0.52)^{l}
31	T83	13.33 (21.41)^{ghijk}	0.00 (0.52)^{i}	6.67 (14.96)^{mn}	3.33 (10.51)^{kl}
32	T220	100.00 (90.00)^{a}	100.00 (90.00)^{a}	76.67 (61.18)^{c}	60.00 (50.77)^{de}
33	T250	100.00 (90.00)^{a}	100.00 (90.00)^{a}	76.67 (61.18)^{c}	56.67 (48.83)^{e}
34	T353	100.00 (90.00)^{a}	100.00 (90.00)^{a}	73.33 (58.91)^{cd}	63.33 (52.73)^{cde}
35	T355	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}	90.00 (71.56)^{b}
36	T357	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
37	T381	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
38	T385	0.00 (0.52)^{m}	0.00 (0.52)^{i}	13.33 (21.41)^{klm}	3.33 (10.51)^{kl}
39	T386	100.00 (90.00)^{a}	100.00 90.00)^{a}	50.00 (45.00)^{fg}	23.33 (28.88)^{g}
40	T387	93.33 (75.03)^{b}	93.33 (75.03)^{c}	63.33 (52.73)^{de}	40.00 (39.23)^{f}
41	T388	100.00 (90.00)^{a}	100.00 90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
42	T389	96.67 (79.49)^{ab}	50.00 (45.00)^{e}	83.33 (65.90)^{bc}	73.33 (58.91)^{c}
43	T390	30.00 (33.21)^{ef}	16.67 (24.10)^{f}	6.67 (14.97)^{mn}	43.33 (41.17)^{f}
44	T392	40.00 (39.23)^{de}	0.00 (0.52)^{i}	20.00 (26.56)^{jk}	3.33 (10.51)^{kl}
45	T393	0.00 (0.52)^{m}	0.00 (0.52)^{i}	0.00 (0.52)^{o}	0.00 (0.52)^{l}
46	T395	0.00 (0.52)^{m}	3.33 (10.51)^{hi}	6.67 (14.97)^{mn}	0.00 (0.52)^{l}
47	T396	3.33 (10.51)^{lm}	0.00 (0.52)^{i}	6.67 (14.97)^{mn}	3.33 (10.51)^{kl}
48	T397	13.33 (21.41)^{ijk}	0.00 (0.52)^{i}	3.33 (10.51)^{no}	0.00 (0.52)^{l}

Table 5 (continued)

Sl. No	*Bt* isolates	Larval mortality (%) 72 h after treatment (20 µg/ml)			
		P. xylostella	*H. armigera*	*S. litura*	*S. frugiperda*
49	T398	23.33 (28.88)^{fgh}	6.67 (14.97)^{gh}	6.67 (14.97)^{mn}	0.00 (0.52)^{l}
50	T399	6.66 (14.96)^{kl}	13.33 (21.41)^{fg}	13.33 (21.41)^{mn}	0.00 (0.52)^{l}
51	HD-1	100.00 (90.00)^{a}	100.00 90.00)^{a}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
52	Control	0.00 (0.52)^{m}	0.00 (0.52)^{i}	0.00 (0.52)^{o}	0.00 (0.52)^{l}
SEd		4.9779	4.1682	3.8622	3.7000
CD (0.05)		9.8720	8.2662	7.6593	7.3377

Figures in parentheses are arcsine transformed values of percentages. Values followed by the same letters in a column are not significantly different ($p = 0.05$) by DMRT

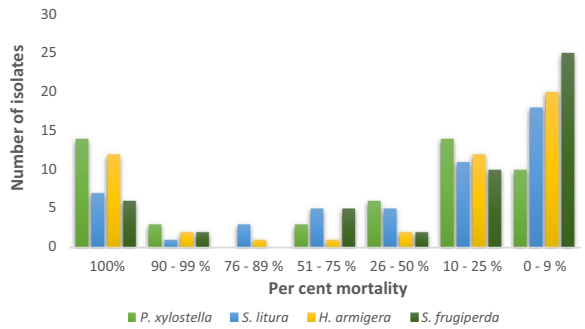


Fig. 4 Variation in mortality of lepidopteran larva with 20 µg/ml of spore crystal mixtures of indigenous *Bt* isolates

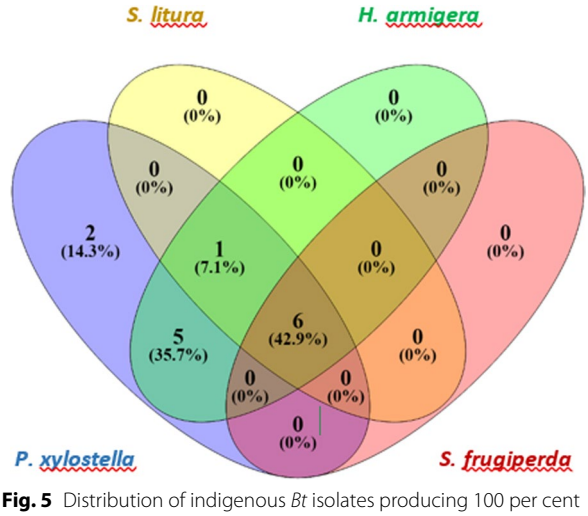


Fig. 5 Distribution of indigenous *Bt* isolates producing 100 per cent mortality in four species of lepidopteran insects

one species of insect. Similar results were reported earlier, and they found spore crystal mixtures of *Bt* isolates able to cause 100% mortality in *P. xylostella* (Navya et al. 2021), *H. armigera* (Lone et al. 2017), *S. litura* (Nayanganesh et al. 2018) and *S. frugiperda* (Karuppaiyan et al. 2022). In this present study, diversified larval mortality was observed among the *Bt* isolates screened against the test insects. These results revealed that the *Bt* isolates are not always effective against all the lepidopteran insects, especially the species of *Spodoptera,* which could be due to natural resistance developed against specific proteins. *S. frugiperda* populations showed to be naturally resistant to the *Bt* proteins Cry1Ab and Cry1Ac (Huang et al. 2014). The fall armyworm population from Puerto Rico exhibited stable Cry1F and Cry1Ac resistance but was still vulnerable to Cry1A.105 and Cry2Ab2 proteins (Gutierrez-Moreno et al. 2020). Besides, the *Bt* isolates with *cry1, cry2,* and *vip3* gene combinations produced 100% toxicity to all the tested insects, as reported earlier by Maheesha et al. (2021) in *S. frugiperda*. Federici (1999) reported that *Bt* products are not always effective against certain noctuid pests, especially *S. litura, S. frugiperda,* and *S. littoralis*, whereas Sahin et al. (2018) reported that Cry1 and Cry2 type proteins are toxic to many insects viz., *S. exigua, S. littoralis, S. frugiperda, H. armigera,* and *Grapholita molesta*. Hence, the observed toxicity of tested *Bt* isolates against lepidopteran insects is presumed to be mainly due to the expression of Cry/Vip proteins.

Conclusion

The present investigation revealed the diversity among the indigenous *Bt* isolates in terms of colony and crystal morphology, protein content, gene content, and toxicity to lepidopteran pests. Six out of 50 isolates produced 100% larval mortality against all the tested species, viz. *P. xylostella, H. armigera, S. litura,* and *S. frugiperda*. These results suggest that some of these isolates after proper evaluation of toxicity, the spectrum of activity, and speed of kill against a different group of lepidopteran insects can be developed into broad-spectrum biopesticides. Although some of the isolates had more than one *cry/vip* gene did not produce any toxic protein; some of the *Bt* isolates did not produce any amplicon with the primer

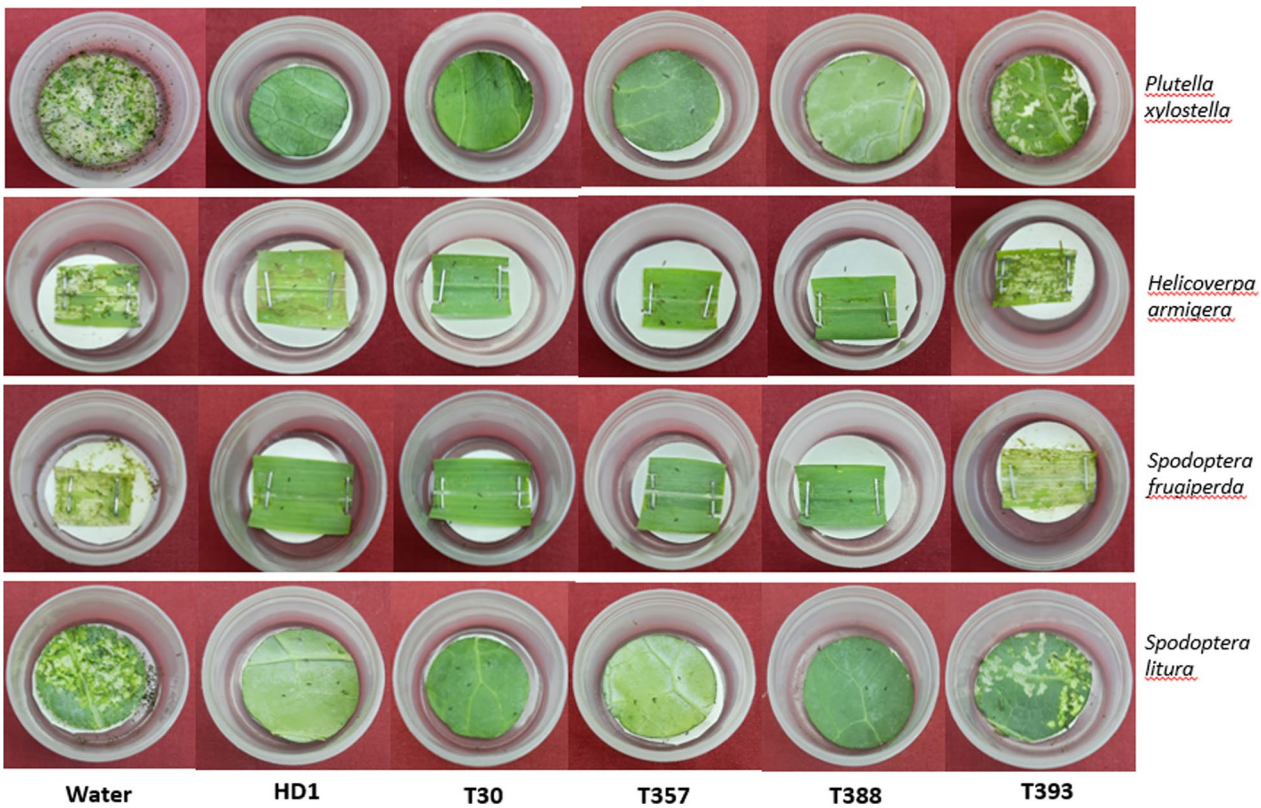


Fig. 6 In vitro insect bioassay with spore-crystal mixtures of *Bt* isolates against key lepidopteran pests. Negative control—water; positive control—HD1; toxic *Bt* isolates—T30, T357 and T388; non-toxic isolate—T393

sets used but produced distinct protein bands; some of the isolates showed distinct protein bands but did not show any toxicity; all these necessitates further investigations for the presence of novel *cry* genes and novel protein.

Abbreviations

Bt: *Bacillus thuringiensis*; °C: Degree centigrade; RH: Relative humidity; pH: Potential of hydrogen; rpm: Revolution per minute; h: Hour/hours; min: Minutes; g: Gram/grams; µg: Microgram; ml: Millilitres; µl: Microlitres; M: Molar; mM: Millimolar; µM: Micromolar; cm: Centimetres; UV: Ultraviolet; PCR: Polymerase chain reaction; dNTPs: Deoxynucleotide triphosphates; DNA: Deoxyribonucleic acid; Kb: Kilo base; bp: Base pair; kDa: Kilo Dalton.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-022-00639-y.

Additional file 1: **Table S1**. Details of indigenous *Bt* isolates and reference strains used in this study. **Table S2**. Primers and PCR conditions used in this study. **Table S3**. Cry protein and gene distribution in native *Bt* isolates.

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Author contributions

GR, VB and TT designed the experimental layout. GR performed the experiment. TT, SM and EK helped in the planning of the experiment. VB, EK and SM provided research material and helped in conducting the experiments. GR prepared the manuscript. VB, SM and EK helped in reviewing and editing the manuscript. All authors have read and approved the final manuscript.

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Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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