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# Diversity of indigenous *Bacillus thuringiensis* isolates toxic to the diamondback moth, *Plutella xylostella* (L.) (Plutellidae: Lepidoptera)

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## Abstract

**Background:** Toxins from the *Bacillus thuringiensis* (*Bt*) bacterium are employed as an alternative to synthetic pesticides in pest management. The greatest threat to the long-term viability of *Bt* toxins is resistance evolution in the target pests. Genetic diversity and toxicity of *Bt* isolates were studied in this work in order to find *Bt* isolates with novel *cry* genes.

**Results:** In terms of colony morphology, among a total of 60 isolates, 51 isolates had off-white colour colonies with typical fried egg appearance, irregular shape, flat and undulate margin. Different crystal shapes, viz. spherical (88.13%), bipyramidal (49.15%), cuboidal (42.37%), rectangular, and crystals attached to spores (3.38%) were observed among *Bt* isolates. SDS-PAGE analysis of spore crystal mixture showed the presence of proteins with various molecular weights ranging from 124 to 26 kDa. PCR screening with *cry1*, *cry2*, *cry9* and *vip3A1* primers showed isolates with varied insecticidal gene combinations. *Bt* isolates containing *cry1* genes were found to be abundant (30), followed by *cry2* (9) and *vip3A1* (9). *Cry9* was absent in all the 60 isolates tested. Insecticidal activity of spore crystal mixtures ranged from 0 to 100% mortality. Furthermore, 12 isolates were found to be highly toxic against the larvae of diamondback moth, *Plutella xylostella* (L.) (Plutellidae: Lepidoptera) with 100% mortality, at 25 µg/ml in leaf disc bioassay.

**Conclusions:** The present work established the diversity of *Bt* isolates and confirmed the importance of continuous exploration of new *Bt* isolates for novel genes. Further, research needs to be carried out to unveil the hidden potential of these toxic isolates.

**Keywords:** *Bacillus thuringiensis*, Diversity, *Plutella xylostella*, Genes, Mortality, Crystal, Morphology toxicity, Toxins

## Background

The most widely used biopesticide, *Bacillus thuringiensis* (Berliner) (Bacillaceae: Bacillales) (*Bt*), is a gram-positive, spore-forming entomopathogenic bacterium usually found in soil, leaf surfaces, grain dusts, dead insects and aquatic environments (Gupta et al. 2021). *Bt* can be distinguished from other *Bacillus* spp. due to its capacity to produce insecticidal proteins during sporulation phase as parasporal inclusions, which predominantly comprises of Cry and Cyt toxins (Schnepf et al. 1998). Numerous *Bt* strains that showed toxic activity towards wide range of insects including Lepidoptera, Coleoptera, Diptera, Homoptera, Mallophaga, Hymenoptera and Nematodes (Sandeep et al. 2020) have been isolated and eventually used to develop biopesticides. At present, 400 *Bt*-based formulations made up of insecticidal proteins and spores have been registered in the market. These toxins are employed in pest management in the form of foliar sprays or by delivering them to the target insect through the expression of toxin encoding genes in the transgenic plants (George and Crickmore 2012). Biopesticides are applied at 10–50 g/acre or $10^{20}$ molecules/

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acre, while chemical pesticides such as organophosphates and pyrethroids are applied at $8 \times 10^{24}$ and $3 \times 10^{22}$ molecules/acre, respectively. By this, the molecular potency of *Bt* toxins is 80,000 and 300 times higher than organophosphates and pyrethroids (Singh et al. 2021). Based on the amino acid sequence homology, 80 holotypes of crystal proteins (*Cry*1–*Cry*80), 4 holotypes of vegetative insecticidal proteins (Vip1–Vip4) and 7 holotypes of cytolytic proteins (Cyt1–Cyt7) of *Bt* have been identified (Crickmore et al. 2018). *Cry* proteins are highly selective to their target insect, innocuous to humans, non-target pests, vertebrates and rapidly degrade in the environment. Therefore, *Bt* is an effective alternative for the control of insect pests in agriculture. However, the primary disadvantage in the long-term use of *Bt* toxins contribute in decreasing susceptibility of pest population. It has been shown that insects developed resistance to *Bt* toxins in the laboratory and in field to *Bt* sprays (Jurat-Fuentes et al. 2021). As a result, isolation of *Bt* strains with novel toxins has gained significant importance for providing alternatives to these problems. With this background, the present study was carried out to screen novel *Bt* isolates that could be toxic to the diamondback moth, *P. xylostella* larvae under controlled conditions.

## Methods

### Insect culture

A laboratory culture of *P. xylostella* was established initially from pupae obtained from National Bureau of Agricultural Insect Resources, Bangalore, India. The pupae were transferred to the rearing cages ($45 \times 45 \times 45$ cm) for the emergence of adults. The newly emerged adults of *P. xylostella* were fed on 10% sugar solution, and mustard seedlings were provided as oviposition substrate and the hatched larvae were fed by fresh cauliflower leaves (CFL-1522, Syngenta) maintained under greenhouse conditions without any insecticide application. The larvae were placed in a ($30 \times 30 \times 30$ cm) larval rearing cage with a glass top, wooden bottom and mesh on all 4 sides. The insect culture was maintained at the Insect Bioassay laboratory (Department of Plant Biotechnology, TNAU, Coimbatore, India) under controlled conditions of temperature, humidity and photoperiod ($25 \pm 1$ °C, $75 \pm 5$% RH and 16: 8 h (L: D).

### *Bt* isolates and growth conditions

Sixty native *Bt* isolates were obtained from Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, TNAU, Coimbatore, India. The reference strain *Bacillus thuringiensis* subsp. *Kurstaki* HD1 originally obtained from *Bacillus* Genetic Stock Center (Columbus, Ohio) was used. In order to obtain single colonies, all the isolates were subcultured on T3 agar media plates using the quadrant streak method and incubated at 30 °C for 12–14 h. Single colony was picked using sterile loop and transferred to the test tube containing 5 ml of T3 broth, incubated at 30 °C for 24 h at 200 rpm and stored as sterile 50% glycerol stocks at − 20 °C for further use.

### Isolation of spore–crystal mixture for toxicity analysis

The spore crystal mixture was isolated from 58 *Bt* isolates and a reference strain, HD1 as a positive control and an acrystalliferous strain 78/11 as a negative control. To obtain the spore crystal mixture from each isolate, a single colony of *Bt* culture from T3 agar plates was inoculated into culture tubes containing 5 ml of T3 broth and kept for overnight incubation in a shaking incubator (Orbitek, Scigenics Biotech Pvt. Ltd, Chennai, India) maintained at 30 °C and 200 rpm. From the overnight grown cultures, 1% inoculum (250 µl) was added to the 250-ml conical flask containing 25 ml of T3 broth in a shaking incubator maintained at 30 °C, 200 rpm for 48–60 h. The growth and lysis of the bacterial cells were checked using the phase contrast microscope after 48 h. When more than 90% cells have lysed, culture was centrifuged at 4 °C for 10 min at 10,000 rpm (Centrifuge 5810R, Eppendorf, Germany) and resulting pellet was suspended in 25 ml of ice-cold Tris–EDTA buffer [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM phenyl methyl sulphonyl fluoride (PMSF)] and washed once with 25 ml of ice-cold 0.5 M NaCl and centrifuged for 10 min, followed by 2 washes with 25 ml Tris–EDTA buffer with 0.5 mM PMSF at the same speed and time (Ramalakshmi and Udayasuriyan 2010). Finally, the pellet was suspended in 500 µl of sterile distilled water containing 10 µl of 1 mM PMSF and stored at − 20 °C as aliquots of 50 µl for later use.

### Colony and crystal morphology

Colony morphology pertaining to colour, surface and margin of each single bacterial colony was observed from the culture plate maintained at 30 °C for 24 h. To examine the shape of the parasporal crystalline inclusions using glass slides, culture smears were prepared on a glass slide, heat fixed and stained with Coomassie brilliant blue stain (0.133% Coomassie Brilliant Blue G250 in 50% acetic acid) for about one min. Smears were washed gently in running water, blot-dried and observed through the phase contrast microscope.

### Protein analysis

Protein analysis using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was done for spore crystal mixture from all the *Bt* samples. SDS-PAGE was performed by following the standard protocol (Laemmli 1970), using 10% separating gel and 4% stacking gel.

The spore crystal mixtures were mixed by loading buffer (4x) containing 0.25 M Tris HCl pH 6.8, 8% SDS, 40% glycerol, 0.5% bromophenol blue in the ratio 4:1, respectively. Then, samples were boiled for 2 min before loading into wells. The molecular mass of the proteins was estimated using pre-stained three-colour protein ladder (Puregene, Genetix Biotech Asia Pvt. Ltd.) covering a wide range molecular weight from 10 to 315 kDa.

#### Detection of *cry* genes in *Bt* isolates

Total genomic DNA was isolated from a single bacterial colony of all isolates according to method described by Sambrook and Russell (2001) and used as a template for the amplification of *cry1*, *cry2*, *cry9* and *vip3A1* genes. The isolated DNA was quantified through NanoDrop (Genova Nano, Jenway), and the integrity was evaluated by agarose gel electrophoresis (1%). Each polymerase chain reaction (PCR) mixture contained 20–50 ng template DNA, 1 µM of each primer (Ben-Dov et al. 1997) and 10 µl of 2 × PCR Master Mix (Smart prime) consisting of dNTPs, Taq polymerase and PCR buffer, and the final volume was made up to 20 µl with sterile distilled water. PCR amplification was performed in a thermal cycler (ProFlex PCR system, Applied Biosystems). The PCR amplicons were separated in 1% agarose gel using 1 kb DNA ladder. The amplified products were visualized under UV transilluminator (Bio-Rad).

#### Insect toxicity assays with *Bt* isolates

Insecticidal activity of the spore crystal mixtures of all the *Bt* isolates was tested against 3-day-old *P. xylostella* larvae. In order to conduct the bioassay, the protein concentration was estimated using Bradford method (Bradford 1976) and the suspension was diluted to 25 µg/ml of concentration in all the isolates before conducting toxicity tests. In all the cases, spore crystal mixture was smeared on small discs (25 mm diameter) of cauliflower leaves and allowed to dry. To prevent desiccation, all the leaf discs were placed on the wet surface of Whatman filter paper discs in plastic cups (30 mm diameter). For each isolate, 3 replications with 10 larvae per replicate were used. The standard strain, *Bt* subsp. *kurstaki* (*Btk*) HD1 and acrystalliferous strain 78/11 were used as positive and negative controls, respectively. Insect mortality was recorded at 24-h interval for 3 days, and cumulative mortality at 72 h was taken as mortality for comparing isolates.

#### Statistical analysis

Experiments were carried out in a completely randomized design (CRD). Single-dose bioassays were statistically analysed by one-way analysis of variance (ANOVA) using AGRES statistical software version 7.01, and significant differences between means were determined by Duncan's multiple range test ($p = 0.05$).

## Results

#### Characterization based on colony and crystal morphology

*Bt* isolates were characterized based on colony colour, surface, shape, elevation, margin, *cry* gene content and protein profile. Among the 60 isolates, 53 isolates were off-white in colour, whereas 7 were full white. Colony surface was found to be fried egg type with flat elevation and undulate margin in 51 isolates, while smooth, raised and entire in 9 isolates. The colony shape of 53 isolates appeared as irregular whereas circular in 7 isolates (Table 1). Phase contrast microscopic (100x) observations (Iscope, Euromex) showed the presence of spores and crystal inclusions. Out of 51 isolates, crystal shape appearance was varied from spherical (88.13%), bipyramidal (49.15%), cuboidal (42.37%), rectangular (3.38%) and minute crystals attached to spores (3.38%) (Table 2).

#### Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of parasporal crystal proteins revealed various banding patterns among the *Bt* isolates. *Bt* isolates carried proteins of different molecular weights, viz. ~124 kDa (20 isolates), ~90 kDa (8 isolates), ~70 kDa (50 isolates), ~60 kDa (2 isolates), ~55 kDa (2 isolates), ~45 kDa (11 isolates) and ~26 kDa (15 isolates) (Table 3).

#### Distribution of lepidopteran specific *cry* and *vip* genes in *Bt* isolates

PCR analysis for four lepidopteran specific genes (*cry1*, *cry2*, *cry9* and *vip3A1*) showed the presence of only *cry1*, *cry2* and *vip3A1* genes but not *cry9* gene. Out of 51 isolates, *cry1*, *cry2* and *vip3A1* gene were found to be present in 30, 9 and 9 isolates, respectively. Fourteen isolates showed the presence of more than one gene, in different combinations (Table 3).

#### Insecticidal activity against *P. xylostella* larvae

From the toxicity results, it was evident that 12 *Bt* isolates, viz. RM12, RM17, RM22, RM29, T15, T20, T29, T32, T152, T191, T380 and T405, showed 100% mortality on par with the positive standard check, HD1 (Tables 4 & 5) after 72 h, against 3-day-old larvae of *P. xylostella*. Two isolates; RM14, T354 exhibited 90 and 93.33% mortality, respectively. *Bt* isolates RM3, RM27, RM28, VB7 and VB16 showed a very least toxicity (< 10%) against *P. xylostella* larvae.

**Table 1** Colony morphology of indigenous *Bacillus thuringiensis* isolates

| Colony parameters | Name of *Bacillus thuringiensis* isolates (n = 60) |
|---|---|
| *Colour* | |
| Full white (7) | RM1, RM5, RM7, RM8, RM13, RM28, RM1R1 |
| Off-white (53) | RM2, RM3, RM6, RM9, RM10, RM11, RM12, RM14, RM15, RM17, RM18, RM19, RM20, RM21, RM22, RM23, RM34, RM25, RM26, RM27, RM29, RM30, RM1R2, T15, T16, T20, T29, T32, T152, T191, T210, T354, T380, T405, VB1, VB2, VB3, VB4, VB5, VB6, VB7, VB8, VB9, VB10, VB11, VB12, VB16, VB17, VB18, VB19, VB20, HD1, 78/11 |
| *Surface* | |
| Fried egg (51) | RM2, RM3, RM6, RM9, RM10, RM11, RM12, RM14, RM15, RM17, RM18, RM19, RM20, RM21, RM22, RM23, RM34, RM25, RM26, RM27, RM29, RM30, RM1R2, T15, T16, T29, T32, T152, T191, T210, T354, T380, VB1, VB2, VB3, VB4, VB5, VB6, VB7, VB8, VB9, VB10, VB11, VB12, VB16, VB17, VB18, VB19, VB20, HD1, 78/11 |
| Smooth (9) | RM1, RM5, RM7, RM8, RM13, RM28, RM1R1, T20, T405 |
| *Shape* | |
| Irregular (53) | RM2, RM3, RM6, RM9, RM10, RM11, RM12, RM14, RM15, RM17, RM18, RM19, RM20, RM21, RM22, RM23, RM34, RM25, RM26, RM27, RM29, RM30, RM1R2, T15, T16, T20, T29, T32, T152, T191, T210, T354, T380, T405, VB1, VB2, VB3, VB4, VB5, VB6, VB7, VB8, VB9, VB10, VB11, VB12, VB16, VB17, VB18, VB19, VB20, HD1, 78/11 |
| Circular (7) | RM1, RM5, RM7, RM8, RM13, RM28, RM1R1 |
| *Elevation* | |
| Raised (9) | RM1, RM5, RM7, RM8, RM13, RM28, RM1R1, T20, T405 |
| Flat (51) | RM2, RM3, RM6, RM9, RM10, RM11, RM12, RM14, RM15, RM17, RM18, RM19, RM20, RM21, RM22, RM23, RM34, RM25, RM26, RM27, RM29, RM30, RM1R2, T15, T16, T29, T32, T152, T191, T210, T354, T380, VB1, VB2, VB3, VB4, VB5, VB6, VB7, VB8, VB9, VB10, VB11, VB12, VB16, VB17, VB18, VB19, VB20, HD1, 78/11 |
| *Margin* | |
| Entire (9) | RM1, RM5, RM7, RM8, RM13, RM28, RM1R1, T20, T405 |
| Undulate (51) | RM2, RM3, RM6, RM9, RM10, RM11, RM12, RM14, RM15, RM17, RM18, RM19, RM20, RM21, RM22, RM23, RM34, RM25, RM26, RM27, RM29, RM30, RM1R2, T15, T16, T29, T32, T152, T191, T210, T354, T380, VB1, VB2, VB3, VB4, VB5, VB6, VB7, VB8, VB9, VB10, VB11, VB12, VB16, VB17, VB18, VB19, VB20, HD1, 78/11 |

* HD1 and 78/11 are standard strains

**Table 2** Crystal morphology of *Bacillus thuringiensis* isolates

| Crystal shape | Number of isolates (Total = 59) | % Occurrence |
|---|---|---|
| Bipyramidal | 29 | 49.15 |
| Spherical | 52 | 88.13 |
| Cuboidal | 25 | 42.37 |
| Rectangular | 2 | 3.38 |
| Crystal attached to spore | 2 | 3.38 |

**Table 3** Crystal protein profile and *cry* genes distribution in native *Bt* isolates

| Parameters | Variations observed | No. of isolates (out of 59) |
|---|---|---|
| Protein size | ~124 kDa | 20 |
| | ~90 kDa | 8 |
| | ~70 kDa | 50 |
| | ~60 kDa | 2 |
| | ~55 kDa | 2 |
| | ~45 kDa | 11 |
| | ~26 kDa | 15 |
| Number of protein bands in SDS-PAGE | One | 22 |
| | Two | 25 |
| | Three | 11 |
| | Four | 1 |
| Number of PCR-positive isolates for *cry* genes | *cry1* | 30 |
| | *cry2* | 9 |
| | *cry9* | 0 |
| | *vip3* | 9 |
| | *cry1* + *cry2* | 10 |
| | *cry1* + *cry2* + *vip3* | 5 |
| | *cry1* + *vip3* | 9 |
| | *cry2* + *vip3* | 5 |

## Discussion

*Bt* collections exhibiting distinct level of toxicity to different insect pests are a good source of novel genes encoding for insecticidal proteins which could be employed in pest management. *Bt* isolates featured matt to granular surface textures with circular to irregular colonies and entire or undulate edges (Logan and De Vos 2011). *Bt* formed colonies on agar plates giving fried egg appearance as reported earlier (Zothansanga et al. 2016). The presence of parasporal crystalline inclusions is the common way to identify *Bt* (Rampersad and Ammons 2005). In the present study, spherical crystals were found in more frequency (88.13%) than bipyramidal crystals

**Table 4** Mortality of diamondback moth, *Plutella xylostella* caused by indigenous *Bt* isolates

| Treatments | Mortality (%) | S. no | Treatments | Mortality (%) | S. no | Treatments | Mortality (%) |
|---|---|---|---|---|---|---|---|
| RM1 | 36.7 (37.3)$^{hijklm}$ | 21 | RM23 | 30.0 (33.2)$^{jklm}$ | 41 | T405 | 100.0 (90.0)$^{a}$ |
| RM2 | 50.0 (45.0)$^{fghi}$ | 22 | RM24 | 43.3(41.2)$^{ghijk}$ | 42 | VB1 | 40.0 (39.2)$^{ghijkl}$ |
| RM3 | 6.7 (15.0)$^{opq}$ | 23 | RM25 | 43.3(41.2)$^{ghijk}$ | 43 | VB2 | 40.0 (39.2)$^{ghijkl}$ |
| RM5 | 66.7 (54.7)$^{def}$ | 24 | RM26 | 56.7 (48.8)$^{def}$ | 44 | VB3 | 26.7 (31.1)$^{klmn}$ |
| RM6 | 70.0 (56.8)$^{cde}$ | 25 | RM27 | 6.7 (15.0)$^{opq}$ | 45 | VB4 | 56.7 (48.8)$^{defg}$ |
| RM7 | 73.3 (58.9)$^{bcd}$ | 26 | RM28 | 10.0 (18.4)$^{nopq}$ | 46 | VB5 | 46.7 (43.1)$^{ghij}$ |
| RM8 | 73.3 (58.9)$^{bcd}$ | 27 | RM29 | 100.0 (90.0)$^{a}$ | 47 | VB6 | 53.3 (46.9)$^{efgh}$ |
| RM9 | 53.3 (46.9)$^{fgh}$ | 28 | RM30 | 36.7 (37.3)$^{hijklm}$ | 48 | VB7 | 3.3 (10.5)$^{pq}$ |
| RM10 | 86.7 (68.6)$^{abc}$ | 29 | RM1R1 | 43.3 (41.2)$^{ghijk}$ | 49 | VB8 | 50.0 (45.0)$^{ghi}$ |
| RM11 | 86.7 (68.6)$^{abc}$ | 30 | RM1R2 | 43.3 (41.2)$^{ghijk}$ | 50 | VB9 | 50.0(45.0)$^{fghik}$ |
| RM12 | 100.0 (90.0)$^{a}$ | 31 | T15 | 100.0 (90.0)$^{a}$ | 51 | VB10 | 43.3 (41.2)$^{ghijk}$ |
| RM13 | 40.0 (39.2)$^{ghjkl}$ | 32 | T16 | 26.7 (31.1)$^{klmn}$ | 52 | VB11 | 33.3 (35.3)$^{ijlm}$ |
| RM14 | 90.0 (71.6)$^{ab}$ | 33 | T20 | 100.0 (90.0)$^{a}$ | 53 | VB12 | 10.0 (18.4)$^{nopq}$ |
| RM15 | 46.7 (43.1)$^{ghij}$ | 34 | T29 | 100.0 (90.0)$^{a}$ | 54 | VB16 | 0.0 (0.5)$^{q}$ |
| RM17 | 100.0 (90.0)$^{a}$ | 35 | T32 | 100.0 (90.0)$^{a}$ | 55 | VB17 | 56.7 (48.8)$^{defg}$ |
| RM18 | 53.7 (47.1)$^{efgh}$ | 36 | T152 | 100.0 (90.0)$^{a}$ | 56 | VB18 | 23.3 (28.9)$^{lmno}$ |
| RM19 | 43.3 (41.2)$^{ghijk}$ | 37 | T191 | 100.0 (90.0)$^{a}$ | 57 | VB19 | 20.0 (26.6)$^{mnop}$ |
| RM20 | 53.3 (46.9)$^{efh}$ | 38 | T210 | 30.0 (33.2)$^{jklm}$ | 58 | VB20 | 20.0 (26.6)$^{mnop}$ |
| RM21 | 43.3 (41.2)$^{gijk}$ | 39 | T354 | 96.7 (79.5)$^{a}$ | 59 | HD1 | 100.0 (90.0)$^{a}$ |
| RM22 | 100.0 (90.0)$^{a}$ | 40 | T380 | 100.0 (90.0)$^{a}$ | 60 | Control | 0.0 (0.5)$^{q}$ |
| SEd | 0.8654 | | | | | | |
| CD (0.05) | 1.7135 | | | | | | |

Figures in parentheses are arcsine transformed values of percentages

Values followed by same letters in a column are not significantly different (0.05) by DMRT

**Table 5** Grouping of *Bt* isolates based on mortality of *Plutella xylostella* larvae at 25 µg/ml

| ***Bacillus thuringiensis* isolates** | **Mortality range (%)** |
|---|---|
| RM12, RM17, RM22, RM29, T15, T20, T29, T32, T152, T191, T380, T405 | 100 |
| RM14, T354 | 90–99 |
| RM7, RM8, RM10, RM11 | 70–89 |
| RM5, RM6, RM20, RM26, VB4, VB6, VB8, VB9, VB17 | 50–69 |
| RM1, RM2, RM9, RM13, RM19, RM21, RM23, RM24, RM25, RM30, RM1R1, RM1R2, T210, VB5, VB11 | 30–49 |
| RM15, RM18, T16, VB1, VB2, VB3, VB10, VB12, VB18, VB19, VB20 | 10–29 |
| RM3, RM27, RM28, VB7, VB16 | 0–9 |

(49.15%). These findings are in accordance with reports by Reyaz et al. (2017) from India and Nair et al. (2018) from Qatar. Apart from these shapes, in the present study cuboidal crystals and crystals attached to spore were found in 42.37 and 3.38% isolates, respectively, as reported earlier by Rampersad and Ammons (2005). On the other hand, Ramalakshmi and Udayasuriyan (2010) observed more frequent occurrence of cuboidal (27%) and bipyramidal crystals (21%) among the 316 *Bt* isolates tested. In terms of crystal morphology, isolates that are considered to be highly toxic produced 2 types of crystals, viz. bipyramidal and cuboidal, which is consistent with previous reports on crystal shape (Boonmee et al. 2019). All these variations in the morphology of crystalline inclusions suggested the presence of diversity in the studied isolates.

Carozzi et al. (1991) introduced PCR-based identification of *cry* genes to predict the insecticidal activity of *Bt* isolates, and it has since become a common tool. In the present study, *cry1* was more prevalent than *cry2* and *vip3A1* genes, in accordance with earlier reports (Lone et al. 2017). Among all the *Bt* isolates, only 16 showed the presence of single gene, while other positive isolates confirmed the presence of both *cry* and *vip* genes in combinations. Earlier, Ben-Dov et al. (1997) reported the tendency of occurrence of *cry1* and *cry2* genes together.

*Bt* isolates containing inter group crystalline protein encoding genes are ideal candidates for the development of broad-spectrum biopesticides. The findings presented here are similar to the previous reports (Sahin et al. 2018). All the 12 isolates that showed 100% mortality including positive control (HD1) were PCR-positive for *cry1*. These

findings are comparable to those of Salama et al. (2015) who confirmed that, most isolates were PCR-positive for *cry1* gene. In the present study, *vip3A1*-positive isolates have been identified to contain *cry1* and *cry2* genes also, in agreement with previous studies (Sahin et al. 2018).

SDS-PAGE analysis of all the 59 *Bt* isolates revealed the existence of diversity in our collection. Among these, 22 isolates produced one protein band only while 25 isolates produced 2 separate protein bands with varying molecular weights. Out of 59 *Bt* isolates, 50 produced proteins of ~70 kDa, as reported earlier by Reyaz et al. (2017). The bands observed at 124 kDa were next major proteins in the present study. Thirty *Bt* isolates produced protein bands in the range of 26 to 60 kDa. This wide range of protein banding pattern in these isolates suggested the variable biological activity and specificity for different insect pests. Ramalakshmi and Udayasuriyan (2010) found 2 protein bands of ~135 and ~65 kDa along with proteins of 95, 43 and 30 kDa in their *Bt* isolates. Diverse electrophoretic patterns in Cry proteins with molecular weights ranging from 20 to 160 kDa were reported earlier by Swamy et al. (2013).

Analysis of insecticidal activity of spore/crystal mixtures is an effective way to identify native *Bt* isolates with entomocidal properties (Sahin et al. 2018). In this study, 12 isolates were found to be highly toxic causing 100% mortality to *P. xylostella* larvae after 3 days. Kahrizeh et al. (2017) reported that 9 out of 148 isolates were highly toxic to *P. xylostella* larvae exhibiting 78.3 to 100% mortality. Monnerat et al. (2007) reported that 20 out of 1400 *Bt* isolates were found to be 100% effective to 1-day-old 2$^{nd}$ instar *P. xylostella* larvae after 5 days in selective bioassays. Among a total of 104 *Bt* isolates, 13isolates caused mortality that ranged between 30 and 100% against *P. xylostella* neonates (Silva et al. 2012).

## Conclusions

The present work showed the diversity among the *Bt* isolates, in terms of protein content, colony and crystal morphology, *cry* gene content and toxicity to *P. xylostella.* The presence of more than one *cry* or *vip* gene in the isolates suggested that they had a high frequency of genetic information exchange. The existence of crystals in isolates that were not PCR-positive with the primer tested suggests that these isolates include *cry*/*cyt* genes that code for insecticidal proteins unique to other target insects from other lepidopteran families. The potency of toxic isolates in the present study (RM12, RM17, RM22, RM29, T15, T20, T29, T32, T152, T191, T380 and T405) to *P. xylostella* may be due to variations in amino acid sequences of *cry* or *vip* proteins, accumulation of *cry* toxins or a combination of these factors. Detailed studies on toxicity, spectrum of activity and speed of kill will help to decide the need for whole genome sequencing of the particular *Bt* isolate, isolation and cloning of novel *cry* genes. Such novel *Cry* proteins could be developed in to a biopesticide formulation for direct use in IPM or in development of newer transgenic crops.

#### Abbreviations

*Bt*: *Bacillus thuringiensis*; Cry: Crystal proteins; Cyt: Cytolytic proteins; Vip: Vegetative insecticidal proteins; dNTPs: Deoxyribo nucleotide triphosphates.

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#### Authors' contributions

VB designed the study. RNSN conducted the experiment. RNSN and VB wrote the manuscript. VB, MR and AL provided research material and helped in conducting the experiments. VB, MR, MM and AL helped in reviewing and editing the manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

Not applicable.

## Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

All the authors give their consent to publish the submitted manuscript as "Original paper" in EJBPC.

#### Competing interests

The authors declare that they have no competing interests.

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