

Potentiality of native *Bacillus thuringiensis* isolates against the destructive pest, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)

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Abstract

The diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is a destructive global pest of cruciferous crops that has evolved resistance to most conventional insecticides, including several *Bacillus thuringiensis* (Bt) based formulations. The limited diversity of Bt toxins currently deployed has accelerated resistance development, emphasizing the urgent need to identify novel indigenous Bt isolates harboring diverse Cry and vegetative insecticidal protein (Vip) profiles for sustainable resistance management. In the present study, 58 indigenous Bt isolates were screened for the presence of lepidopteran-active toxin genes and toxicity against *Plutella xylostella*. Polymerase chain reaction (PCR) assays were employed to detect *cry1*, *cry2*, *cry9*, and *vip3A* genes while protein profiling was performed to evaluate toxin diversity among the isolates. Preliminary screening identified twelve promising isolates, which were further subjected to leaf-disc bioassays at multiple concentrations. Larval mortality data were analysed using probit analysis to estimate median lethal concentrations (LC_{50}) and to assess model fit. Scanning electron microscopy revealed diverse parasporal crystal morphologies, including bipyramidal, cuboidal, and spherical forms. PCR analysis detected *cry1*, *cry2*, and *vip3A* genes in several isolates, whereas *cry9* was not detected. Protein profiling revealed substantial diversity among the isolates. LC_{50} values ranged from 0.07 to 0.44 $\mu\text{g mL}^{-1}$ and were comparable to the reference strain, HD1 (0.06 $\mu\text{g mL}^{-1}$). Among the isolates, RM12, T20B, and T32 exhibited the highest toxicity against *P. xylostella* larvae, although their toxicity did not differ significantly from HD1 based on overlapping 95% fiducial limits. The combined occurrence of multiple *cry* and *vip* genes, together with diverse protein profiles and crystal morphologies, demonstrates considerable genetic and functional diversity among the indigenous Bt isolates, highlighting their potential for the development of effective Bt-based biopesticides against insecticide-resistant *P. xylostella* populations.

Introduction

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is widely regarded as the most destructive insect pest of cruciferous vegetables worldwide, including cauliflower, cabbage and broccoli (Frolov et al. 2025; Li et al. 2016). In the early stages, first-instar larvae mine the leaf tissue, whereas later instars feed on the mesophyll from the abaxial surface, creating characteristic window-like patches that significantly reduce photosynthetic area and commercial value (Kumar et al. 2025). Under continuous cropping systems the pest is capable of completing 10–15 generations annually, and its continuous reproduction leads to persistent and overlapping infestations (Xu et al. 2025). Globally, the economic losses and management costs associated with *P. xylostella* are estimated to be approximately USD 4–5 billion annually (Bermúdez et al. 2024).

The pest inflicts damage throughout the crop growth cycle, from the seedling stage to harvest. Larval feeding causes significant yield losses and further compromises market quality by contaminating the edible portions with frass (Kaur et al. 2021). To mitigate crop losses, farmers frequently rely on frequent and repeated insecticide applications. *P. xylostella* is considered one of the most resistant insect pests globally, ranking first among resistant species and exhibiting resistance to over 104 insecticidal

compounds by Eslamloo and Ito (2025). However, sustained reliance on chemical insecticides, coupled with the pest's genetic plasticity, short life cycle and high fecundity, has led to the evolution of resistance to almost every major insecticide class, including new molecules (Navik et al. 2019; Qu et al. 2026). Moreover, conventional insecticides also pose significant concerns, including toxicity to non-target organisms, residue accumulation in food products, and environmental contamination (Kashyap et al. 2024; Punniyakotti et al. 2024). These limitations underscore the urgent need for environmentally sustainable alternatives that can be effectively incorporated into pest management strategies.

Among biological options, *Bacillus thuringiensis* (Bt) remains one of the most successful and widely used microbial insecticides for controlling lepidopteran pests. Bt is a Gram-positive, spore-forming bacterium that produces parasporal crystalline inclusions during the stationary phase of growth, primarily composed of insecticidal crystal (Cry) proteins (Gonzalez-Vázquez et al. 2021; Bel et al. 2020). When ingested by susceptible larvae, these protoxins are solubilized in the alkaline midgut and subsequently activated by proteolytic cleavage. The activated toxins bind to specific receptors on the midgut epithelial cells, leading to pore formation, disruption of osmotic balance, cell lysis, septicemia and ultimately larval death (Xie et al. 2026; Jurat-Fuentes et al. 2021). Based on amino acid sequence homology, more than 80 Cry holotypes (Cry1 – Cry80), four Vip families (Vip1 – Vip4) and several Cyt proteins have been classified (Crickmore et al. 2016). Hitherto, over 400 Bt based formulations have been registered globally for agricultural use, predominantly as foliar sprays or through their expression into transgenic crops (George and Crickmore 2012).

Bacillus thuringiensis subsp. *kurstaki* (Btk) formulations have been extensively utilized for the management of lepidopteran pests, including *P. xylostella* owing to their demonstrated efficacy under laboratory and field conditions (Zhou et al. 2025). However, prolonged and intensive use of Btk has led to the evolution of resistance, in several regions, thereby reducing its field effectiveness (Gassmann and Reisig 2023).

In addition to widespread resistance to synthetic insecticides, several lepidopteran pests, including *P. xylostella*, have developed resistance to Bt toxins both under laboratory and field conditions. A recent global review compiling data from over 160 studies across 20 countries highlights multiple cases of field-evolved resistance, cross-resistance, and diverse resistance mechanisms, raising concerns about the long-term sustainability of Bt-based pest management strategies (Afzal et al. 2024).

Consistent with this, resistance to Bt, particularly Cry1Ac-based formulations and *Bacillus thuringiensis* subsp. *kurstaki* (Btk), has also been widely documented under field conditions, further limiting their long-term effectiveness (Tabashnik and Carrière 2017; Sun et al. 2025).

According to the sequential binding model, Bt Cry protoxins are solubilized and activated in the alkaline insect midgut and subsequently bind to receptors such as cadherin, aminopeptidase-N (APN), and alkaline phosphatase (ALP). This interaction promotes toxin oligomerization, membrane insertion, and pore formation, ultimately leading to epithelial cell lysis (Crickmore 2016).

At the molecular level, resistance to Bt Cry toxins in *P. xylostella* involves reduced toxin binding affinity, altered midgut protease activity affecting toxin activation, and differential gene expression in the larval midgut. Transcriptomic studies have revealed changes in the regulation of genes associated with digestion, metabolism, and defense responses. Additionally, hormonal feedback pathways involving juvenile hormone and ecdysteroids have been implicated, indicating a complex regulatory network underlying resistance (Crickmore 2016; Xiong et al. 2021; Sun et al. 2025).

The widespread and repeated use of Btk formulations, particularly those based on Cry1Ac toxins, exerts strong selection pressure on pest populations, thereby accelerating resistance evolution (Tabashnik and Carrière 2017). A key limitation of conventional Btk products is their reliance on a narrow spectrum of Cry toxins, which increases the likelihood of cross-resistance (Crickmore 2016). In contrast, indigenous Bt isolates often harbor diverse combinations of *cry* and *vip* genes, producing multiple toxins with distinct modes of action. Notably, Vip proteins differ from Cry toxins in receptor binding and mode of action, making them effective against Cry-resistant populations (Crickmore et al. 2021). Therefore, the use of indigenous Bt isolates with varied toxin profiles offers a promising strategy for resistance management and sustainable pest control. This has spurred global interest in discovering novel Bt strains harboring unique combinations of *cry* and *vip* genes, with improved toxicity and broader insecticidal spectra that can serve as potential alternatives to Cry1Ac-based Btk formulations (Gupta et al. 2021; Aswathi et al. 2024). Studies have demonstrated that Bt isolates exhibit considerable genetic diversity, with variations in *cry* and *vip* genes, resulting in differences in protein profiles, crystal morphology and insecticidal activity (Sahin et al. 2018; Syed et al. 2020).

India, a major producer of cruciferous vegetables, represents a significant hotspot for *P. xylostella* due to its diverse agroecological conditions and continuous host availability (Kaur et al. 2021; Navik et al. 2019). The large-scale and intensive cultivation of crucifer crops in the country plays a critical role in driving the evolution and spread of resistance in this pest. Production systems are typically characterized by frequent insecticide applications and continuous cropping, which impose strong selection pressure on pest populations (Navik et al. 2019). Consequently, field populations of *P. xylostella* in India have developed widespread resistance to multiple insecticide classes, including newer chemistries such as diamides, resulting in reduced field effectiveness (Tamilselvan et al. 2021; Pasupathi et al. 2022). In addition to resistance to synthetic insecticides, *P. xylostella* has also been reported to develop resistance to *B. thuringiensis* toxins, with reduced susceptibility observed in field populations under intensive pest management conditions in India (Bhardwaj et al. 2022).

In this context, indigenous *B. thuringiensis* isolates represent a valuable resource, as they often possess unique combinations of *cry* and *vip* genes with potential for enhanced insecticidal activity and resistance management. In India, diverse agroecological regions harbor a wide range of Bt isolates with distinct toxin gene profiles. Navya et al. (2021) reported considerable diversity among indigenous Bt isolates toxic to *P. xylostella*, suggesting the presence of strains with novel insecticidal potential.

Building on this premise, the present study aimed to evaluate indigenous Bt isolates for their genetic diversity, protein profiles, and insecticidal activity against *P. xylostella*. We hypothesized that indigenous Bt isolates harbor distinct combinations of *cry* and *vip* genes associated with enhanced insecticidal efficacy compared to the reference strain HD1, thereby offering potential alternatives for resistance management. The novelty of this study lies in the comprehensive characterization of indigenous isolates through morphological, molecular, and bioassay approaches to identify potentially toxic strains against *P. xylostella*. These findings contribute to the development of effective and sustainable Bt based biopesticide formulations for managing *P. xylostella*, particularly in regions where resistance to existing Bt products is prevalent.

Materials & Methods

Bt Isolates and Culture Growth Conditions

A total of 58 indigenous *B. thuringiensis* isolates were obtained from the Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University (TNAU), Coimbatore, India (Navya et al. 2021). These isolates were originally collected from soil samples, subsequently purified, and maintained in the TNAU microbial repository. The isolates had not been previously characterized for their genetic or insecticidal properties, and the codes used in this study correspond to their internal laboratory identification. The isolates are currently maintained at TNAU and have not been deposited in a public microbial culture collection. The reference strain *Bacillus thuringiensis* subsp. *kurstaki* HD1 (BGSC accession number: 4D1) obtained from the Bacillus Genetic Stock Center (Columbus, Ohio, USA) was used as a positive control. An acrySTALLiferous *B. thuringiensis* strain (78/11), lacking insecticidal crystal (Cry) proteins, was used as a negative control. Both control strains were maintained and cultured under the same laboratory conditions as the test isolates. For long term preservation, all isolates were stored as sterile 50% glycerol stocks at -20°C. For experimental use, isolates were streaked onto T3 agar plates and incubated at 30°C for 12–14 h. A single colony was inoculated into 5 mL T3 broth and incubated at 30°C with shaking at 200 rpm for 24 h. Subsequently, 1% (v/v) of this culture was transferred into 25 mL T3 broth in a 250 mL flask and incubated under the same conditions for 48–60 h to obtain sporulated cultures rich in spores and crystals (Navya et al. 2021).

Cultures were grown in T3 medium composed of tryptone (0.3 g), tryptose (0.2 g), yeast extract (0.15 g), Mn Cl₂ (10 µL of 0.05 g/mL), Na H₂ PO₄ (0.69 g), and Na₂ HPO₄ (0.89 g) per 100 mL, with the pH adjusted to 6.8–7.0 prior to sterilization. Aerobic conditions were maintained by continuous shaking at 200 rpm.

Colony Morphology Characterization

The colonial morphology of *B. thuringiensis* isolates was evaluated by streaking onto T3 agar plates and incubating at 30°C for 24–48 h. Colony characteristics, including size, shape, color, margin, elevation, and surface texture, were recorded visually. Observations were performed in three independent

replicates for each isolate. Morphological characterization was conducted following standard bacteriological descriptions as outlined in Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986).

Preparation of Spore-Crystal Toxin Mixtures

A single *B. thuringiensis* (Bt) colony was selected from T3 agar plates and inoculated into culture tubes containing 5 mL of T3 broth. The cultures were incubated overnight in an incubator (Orbitek, Scigenics Biotech Pvt. Ltd, Chennai, India) maintained at 30°C and 200 rpm to obtain a mixture containing spores and crystals from each isolate. Subsequently, from the overnight grown Bt cultures, 1% inoculum (250 µL) was transferred into a 250 mL conical flask containing 25 mL of T3 broth and incubated at 30°C and 200 rpm for 48–60 hours (Navya et al. 2021). The cultures were grown until reaching an optical density of 1.0 at 600 nm (OD₆₀₀). Bacterial growth, sporulation, and cell lysis were monitored after 48 h using a phase-contrast microscope. Cell lysis was considered complete when > 90% of cells appeared lysed, based on visual observation of lysed versus intact cells across at least five different microscopic fields, with three independent replicates per isolate. Once > 90% of the cells had lysed, the culture was centrifuged at 10,000 rpm for 10 min at 4°C (Centrifuge 5810R, Eppendorf, Germany).

The resulting pellet was resuspended in 25 mL of ice-cold (4 °C) Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), followed by a wash with 25 mL of ice-cold 0.5 M NaCl and centrifugation under the same conditions. The pellet was further washed twice with 25 mL of Tris-EDTA buffer containing 0.5 mM PMSF (Navya et al. 2021). Finally, the pellet was resuspended in sterile distilled water (500 µL) supplemented with 1 mM PMSF (10 µL) and stored at -20°C in 50 µL aliquots for future use (Ramalakshmi and Udayasuriyan 2010). The protein concentration of the spore–crystal mixtures was determined using the Bradford method and standardized prior to bioassay.

Scanning Electron Microscopy (SEM)

The morphology of spores and crystals from twelve toxic *B. thuringiensis* isolates (RM12, RM17, RM22, RM29, T15, T20B, T29, T32, T152, T191, T380, and T405) was examined using a scanning electron microscope (FEI Quanta 250) at the Department of Nano Science and Technology, TNAU (Navya et al. 2021). These isolates were selected based on their high insecticidal activity (100 per cent) in preliminary bioassays, presence of *cry1*, *cry2* and *vip3* genes confirmed by PCR, and corresponding protein expression profiles observed in SDS-PAGE, along with the standard strain HD1. A 10 µL aliquot of the spore-crystal mixture was added to 1,500 µL of distilled water and vortexed to disperse cell aggregates. From this suspension, 10 µL of the diluted sample was placed onto a sample holder coated with adhesive carbon tape and allowed to air-dry under light at room temperature (25 ± 2 °C) for 5 to 10 min. After complete drying, samples were sputter-coated with gold for approximately 15 s at a current of 20

mA. The coated samples were then placed in the vacuum chamber of the SEM, and images were captured at 12 kV with a magnification of 10,000× (Navya et al. 2021).

Genomic DNA Isolation, PCR amplification, Sequencing and Analysis

Genomic DNA from each *B. thuringiensis* isolate was extracted from individual colonies following the method described by Sambrook and Russell (2001) without modification and used as a template for the amplification of *cry1*, *cry2*, *cry9*, and *vip3A* genes. Cell lysis was performed using an enzymatic lysis approach with lysozyme (1 mg mL⁻¹) in a resuspension buffer containing Tris–HCl (25 mM), sucrose (25%), and EDTA (25 mM), followed by treatment with TES solution (Tris–HCl 10 mM, EDTA 1 mM, NaCl 100 mM). The concentration of the extracted DNA was quantified using a Nanodrop spectrophotometer (Genova Nano, Genway), and standardized to a final concentration of 50 ng µL⁻¹ for PCR analysis. DNA quality and integrity were confirmed by electrophoresis on a 1% agarose gel. Each PCR reaction (20 µL) contained 20–50 ng of template DNA, 1 µM of each specific primer (Table 1), and 10 µL of 2× PCR Master Mix (Smart Prime) comprising dNTPs, Taq DNA polymerase, and PCR buffer. The reaction volume was adjusted with sterile distilled water. PCR amplification was performed in a thermal cycler (Pro Flex PCR System, Applied Biosystems) following the cycling conditions described in Table 2. Amplified PCR products were resolved on a 1% agarose gel alongside a 1 kb DNA ladder, at 120 V for 60 min, and the fragments were visualized under a UV transilluminator (Bio-Rad) (Navya et al. 2021).

Table 1

Details of primers used for screening *Bt* isolates for lepidopteran specific *cry* and *vip* genes

Primer	Sequence 5' → 3'	Amplicon size (bp)	Reference.
cry1	FP: CATGATTCATGCGGCAGATAAAC	274–277 bp	(Ben-Dov et al. 1997)
	RP: TTGTGACACTTCTGCTTCCCATT		
	RP: GCTGCAGTGCTCTTTCTAAATCATATCTGCC		
	RP: CCTGCAGTCCCACTAAAATTTCTAACACCTACTA		
cry2	FP: GTTATTCTTAATGCAGATGAATGGG	689–701 bp	(Ben-Dov et al. 1997)
	RP: CGGATAAAATAATCTGGGAAATAGT		
	RP: TGGCGTTAACAATGGGGGGAGAAAT		
cry9	FP: CGGTGTTACTATTAGCGAGGGCGG	351–359 bp	(Jain et al. 2017)
	RP: GTTTGAGCCGCTTCACAGCAATCC		
vip3Aa	FP: CCTCTATGTTGAGTGATGTA	1000 bp	
	RP: CTATACTCCGCTTCACTTGA		
	RP: TTACTTAATAGAGACATCGTAAAAA		
Details of gene-specific primers used for screening Bacillus thuringiensis isolates for lepidopteran-specific cry1, cry2, cry9, and vip3Aa genes. The table includes primer sequences (5'-3'), expected amplicon sizes (bp), and corresponding references.			

Table 2
PCR profile for screening of *Bt* isolates for *cry1*, *cry2*, *cry9* and *vip3Aa* genes

Step	<i>cry1</i>	<i>cry2</i>	<i>cry9</i>	<i>vip3Aa</i>
Initial denaturation	94°C for 2 minutes	94°C for 2 minutes	94°C for 2 minutes	94°C for 5 minutes
Denaturation	94°C for 40 seconds	94°C for 40 seconds	94°C for 40 seconds	94°C for 1 minute
Annealing	62°C for 40 seconds	60°C for 40 seconds	58°C for 40 seconds	55°C for 1 minute
Primer extension	72°C for 1 minute	72°C for 40 seconds	72°C for 1 minute	72°C for 40 seconds
Step 2 to 4	30 cycles	30 cycles	30 cycles	35 cycles
Final extension	72°C for 7 minutes	72°C for 10 minutes	72°C for 7 minutes	72°C for 10 minutes
PCR amplification conditions used for screening <i>Bacillus thuringiensis</i> isolates for the presence of <i>cry1</i>, <i>cry2</i>, <i>cry9</i>, and <i>vip3Aa</i> genes. The table presents gene-specific thermal cycling parameters, including initial denaturation, denaturation, annealing, primer extension, number of cycles, and final extension steps.				

PCR-amplified products of selected isolates were purified and subjected to sequencing using gene-specific primers (Bio Kart, Bangalore). Partial sequences of *cry1*, *cry2*, and *vip3* genes were obtained from representative isolates.

The resulting forward and reverse sequences were assembled and edited using BioEdit software (Hall, 1999). The edited sequences were compared against entries in the GenBank database using the BLASTn algorithm available at National Center for Biotechnology Information (Altschul et al. 1990). The finalized sequences were subsequently submitted to GenBank, and accession numbers were obtained.

SDS–PAGE Analysis

Protein profiling of the spore-crystal mixtures from all *B. thuringiensis* isolates was carried out using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), following the standard protocol of Laemmli (1970). A 10% separating gel and a 4% stacking gel were used for the protein separation. Spore-crystal samples were mixed with 4× loading buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, and 0.5% Bromophenol Blue) in a 4:1 ratio and boiled for 2 minutes before loading. Approximately 30 µL of each sample was loaded into each well. Electrophoresis was carried out at a constant voltage of 80 V with a current of 20 mA in the stacking gel, to 30 mA in the resolving gel, until the dye front reached the bottom of the gel. Protein molecular weights were estimated using a pre-stained, three-color protein ladder (Puregene, Genetix Biotech Asia Pvt. Ltd.) with a range of 10–315 kDa. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 for 1 h and destained until distinct protein bands were clearly visualized.

Insect rearing and toxicity bioassays

A laboratory population of *P. xylostella* was established initially from pupae obtained from the National Bureau of Agricultural Insect Resources (NBAIR), Bangalore, India (Navya et al. 2021). The initial population was a known susceptible laboratory strain, and the culture was reared for three generations without exposure to insecticides prior to use in bioassays. Pupae were transferred to rearing cages (45 × 45 × 45 cm) for adult emergence. Freshly emerged adults were provided with a 10% sugar solution, and mustard seedlings were used as an oviposition substrate. The hatched (neonate) larvae were reared on freshly harvested cauliflower leaves (CFL-1522, Syngenta) grown under greenhouse conditions without insecticide application. The culture was maintained in the laboratory under controlled conditions at 25 ± 1°C, 75 ± 5% relative humidity (RH), and a photoperiod of 16:8 h (L:D).

The pathogenic activity of Bt isolates against *P. xylostella* larvae was evaluated against 3 days old larvae using a leaf disc bioassay. Cauliflower leaf discs (25 mm dia.) were washed with distilled water containing 0.1% Triton X-100 and air-dried prior to treatment. The experimental unit consisted of a single treated cauliflower leaf disc placed in a plastic cup (30 mm dia.) containing moistened Whatman filter paper to prevent desiccation. Prior to the bioassay, protein concentrations of spore-crystal mixtures were determined using the Bradford method (Bradford, 1976).

Preliminary screening of all isolates was conducted at a standardized protein concentration of 25 µg mL⁻¹, and isolates causing 100 per cent larval mortality were selected for further detailed bioassays. For detailed LC₅₀ studies, each Bt isolate was tested at 8–9 protein concentrations ranging from 0.016 to 5 µg mL⁻¹ (Table 3). Treated leaf discs were air dried and placed in bioassay cups as described above. *Bacillus thuringiensis* subsp. *kurstaki* (Btk) HD1 and sterile water were included as positive and negative controls, respectively. Each treatment was conducted in three replicates, with 10 larvae per leaf disc, (n = 30 larvae per concentration). Larvae were allowed to feed for 72 h, and mortality was recorded at 24 h intervals. Cumulative mortality at 72 h was used to compare the efficacy of the different Bt isolates. Larval feeding on treated leaf discs was partial and variable, and complete consumption of the discs was not observed. Therefore, the exact dose ingested per larva could not be determined, and LC₅₀ values were expressed in terms of concentration (µg mL⁻¹).

Table 3
Different concentrations of *Bt* isolates used for probit analysis to work out LC₅₀

S. No.	Bt isolates	Concentrations (µg/ml) *
1.	RM12	2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.0315, 0.016
2.	RM17	5, 3.75, 2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625
3.	RM22	2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.0315, 0.016
4.	RM29	5, 3.75, 2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625
5.	T15	5, 3.75, 2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625
6.	T20B	5, 3.75, 2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625
7.	T29	2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.0315, 0.016
8.	T32	2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.0315, 0.016
9.	T152	5, 3.75, 2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625
10.	T191	5, 3.75, 2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625
11.	T380	2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.0315, 0.016
12.	T405	2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.0315, 0.016
13.	HD1	2.5, 1.875, 1.25, 0.625, 0.25, 0.125, 0.0625, 0.0315, 0.016
S. No*: Serial number representing the isolates. Different concentrations of <i>Bacillus thuringiensis</i> isolates used for probit analysis to estimate LC₅₀ values against <i>Plutella xylostella</i> larvae.		

Statistical Analysis

Mortality data were corrected for natural mortality using Abbott's formula prior to analysis. The corrected mortality data were subjected to probit analysis using the software Environmental Protection Agency probit analysis program used for calculating LC/EC values (version 1.5) executed in a DOS box environment. Median lethal concentrations (LC₅₀ and LC₉₅), regression equations, 95% fiducial limits, and chi-square (χ^2) goodness-of-fit values were calculated for each isolate. Degrees of freedom (df) were calculated as the number of concentrations tested minus one (df = 8, based on nine concentrations per isolate). The chi-square (χ^2) values were used to assess the adequacy of model fit, where non-significant values indicating an acceptable fit of the probit regression model.). Statistical differences among isolates were determined by comparing the overlap of 95% fiducial limits of LC₅₀ values. Overlapping fiducial limits were considered not significantly different, whereas non-overlapping limits indicated significant differences at $p < 0.05$.

Results

The twelve *Bacillus thuringiensis* isolates selected for detailed evaluation showed clear differences in colony morphology on T3 agar. Most isolates produced off-white colonies with a typical “fried-egg” appearance, flat elevation, rough texture and undulate margins, while one isolate formed a smooth, raised colony with an entire margin. All isolates displayed irregular colony shapes, indicating morphological variation among strains. Scanning electron microscopy revealed distinct crystal morphologies among the isolates. Eleven isolates (T15, T20B, T29, T32, T152, T191, RM12, RM17, RM22, T380 and T405) produced multiple crystal types, including bipyramidal, cuboidal and spherical forms whereas, RM29 predominantly formed bipyramidal crystals. Isolate T15 consistently produced cuboidal crystals (Fig. 1). Isolates producing bipyramidal crystals were found to harbor *cry1* genes, while cuboidal crystal forming isolates also carried *cry2* genes, as confirmed by PCR and sequence analysis. Several isolates produced multiple crystal types and carried more than one *cry* gene. The relative proportions of different crystal types were not quantified and are therefore presented qualitatively based on microscopic observations. The observed diversity in crystal morphology suggests the presence of multiple Cry and Vip proteins, consistent with molecular and biochemical analyses.

Protein profiling using SDS–PAGE supported this diversity. All isolates exhibited a prominent protein band at approximately 124 kDa corresponding to Cry1 proteins. Additional protein bands at ~ 90 kDa (Vip3A) and ~ 65 kDa (Cry2) were detected in several isolates, indicating the production of multiple toxin proteins (Fig. 2). These findings were consistent with the gene amplification patterns obtained through PCR. PCR screening showed that all twelve isolates carried *cry1*, while *cry2* and *vip3A* were present in six and eight isolates, respectively whereas *cry9* was not amplified in any of the isolates (Fig. 3). Gene combination analysis showed that six isolates harbored *cry1 + cry2*, eight isolates contained *cry1 + vip3A*, and five isolates possessed all three toxin genes (*cry1 + cry2 + vip3A*).

Sequence analysis of PCR-amplified products confirmed the identity of toxin genes. Nucleotide sequences of *cry1*, *cry2*, and *vip3* genes were assembled and edited using BioEdit and compared against sequences available in the NCBI database using BLASTn. The *cry1* gene sequences from isolates RM12, RM17, RM22, RM29, T15, T20B, T29, T32, T152, T191, T380, and T405; *cry2* from T15, T29, T32, T380, and T405; and *vip3* from RM12, RM17, RM22, T15, T29, T32, T152, and T405 showed 99–100% sequence identity with corresponding reference sequences in the NCBI database. The aligned sequences were deposited in GenBank (NCBI), and the accession numbers are provided in Table 4.

Table 4

GenBank accession numbers of *cry1*, *cry2* and *vip3* gene sequences obtained from PCR amplification using gene-specific primers

S. No	Bt isolate	Gene	Accession number
1.	RM12	<i>cry1</i>	OM831235
2.	RM17	<i>cry1</i>	OM831236
3.	RM22	<i>cry1</i>	OM831237
4.	RM29	<i>cry1</i>	OM831238
5.	T15	<i>cry1</i>	OM831239
6.	T20B	<i>cry1</i>	OM831240
7.	T29	<i>cry1</i>	OM831241
8.	T32	<i>cry1</i>	OM831242
9.	T152	<i>cry1</i>	OM831243
10.	T191	<i>cry1</i>	OM831244
11.	T380	<i>cry1</i>	OM831245
12.	T405	<i>cry1</i>	OM831246
13.	T15	<i>cry2</i>	OM995800
14.	T29	<i>cry2</i>	OM995801
15.	T32	<i>cry2</i>	OM995802
16.	T380	<i>cry2</i>	OM995803
17.	T405	<i>cry2</i>	OM995804
18.	RM12	<i>vip3Aa</i>	ON009951
19.	RM17	<i>vip3Aa</i>	ON009952
20.	RM22	<i>vip3Aa</i>	ON045316
21.	T15	<i>vip3Aa</i>	ON045317
22.	T29	<i>vip3Aa</i>	ON045318
23.	T32	<i>vip3Aa</i>	ON045319
24.	T152	<i>vip3Aa</i>	ON045320

S. No*: Serial number representing the isolates. GenBank accession numbers correspond to the nucleotide sequences of *cry1*, *cry2*, and *vip3* genes amplified from *Bacillus thuringiensis* isolates using gene-specific PCR primers. The submitted sequences were deposited in the National Center for Biotechnology Information GenBank database.

S. No	<i>Bt</i> isolate	Gene	Accession number
1.	RM12	<i>cry1</i>	OM831235
25.	T405	<i>vip3Aa</i>	ON045321
S. No*: Serial number representing the isolates. GenBank accession numbers correspond to the nucleotide sequences of <i>cry1</i>, <i>cry2</i>, and <i>vip3</i> genes amplified from <i>Bacillus thuringiensis</i> isolates using gene-specific PCR primers. The submitted sequences were deposited in the National Center for Biotechnology Information GenBank database.			

The presence of multiple toxin gene families in nine isolates suggested broad insecticidal potential, consistent with the observed crystal morphology and protein profiles. Toxicity assays conducted on *P. xylostella* larvae demonstrated clear dose-dependent mortality across all isolates. Mortality increased proportionally with the concentration of spore–crystal preparations (Fig. 4–6).

Probit analysis provided LC_{50} and LC_{95} values, regression estimates, fiducial limits, and chi-square (χ^2) values for each isolate. LC_{50} values ranged from 0.07 to 0.44 $\mu\text{g mL}^{-1}$, while the reference strain HD1 recorded an LC_{50} of 0.06 $\mu\text{g mL}^{-1}$. Among the isolates, RM12 (LC_{50} : 0.07 $\mu\text{g mL}^{-1}$; 95% FL: 0.05–0.09), T20B (LC_{50} : 0.09 $\mu\text{g mL}^{-1}$; 95% FL: 0.06–0.13), and T32 (LC_{50} : 0.09 $\mu\text{g mL}^{-1}$; 95% FL: 0.05–0.13) showed overlapping fiducial limits with HD1 (LC_{50} : 0.06 $\mu\text{g mL}^{-1}$; 95% FL: 0.04–0.08), indicating statistically comparable toxicity. The remaining isolates exhibited LC_{50} values ranging from 0.10 and 0.44 $\mu\text{g mL}^{-1}$ with relatively wider fiducial limits, reflecting moderate but consistent insecticidal activity. All probit regressions showed non-significant χ^2 values (df = 8), confirming a good fit of the model (Table 5). Isolates harboring multiple toxin gene families generally exhibited lower LC_{50} values and narrower fiducial limits, indicating higher potency and greater consistency in insecticidal performance.

Table 5
Toxicity of twelve *Bacillus thuringiensis* isolates to *Plutella xylostella* larvae in LC₅₀ assay

Isolate	χ^2 (df) (p > 0.05)	LC ₅₀ ($\mu\text{g mL}^{-1}$)	Fiducial limits (95%)		LC ₉₅ ($\mu\text{g mL}^{-1}$)	Fiducial Limits (95%)	
			Lower limit	Upper limit		Lower limit	Upper limit
RM12	0.62 (8)	0.07	0.05	0.09	0.7	0.41	1.67
RM17	5.61 (8)	0.43	0.27	0.62	9.16	4.78	29.09
RM22	0.72(8)	0.16	0.06	0.29	7.04	3.33	32.63
RM29	2.86 (8)	0.44	0.3	0.61	5.78	3.23	16.12
T15	1.73 (8)	0.27	0.14	0.41	7.29	3.73	25.22
T20B	1.59 (8)	0.09	0.06	0.13	1.56	0.76	5.6
T29	1.26 (8)	0.1	0.07	0.16	2.27	0.98	10.7
T32	1.21 (8)	0.09	0.05	0.13	2.78	1.4	8.24
T152	0.73 (8)	0.44	0.25	0.71	14.8	4.93	252.74
T191	5.83 (8)	0.28	0.1	0.47	15.73	5.42	232.13
T380	4.42 (8)	0.11	0.09	0.15	0.81	0.53	1.52
T405	0.43 (8)	0.1	0.06	0.15	2.19	0.95	10.39
HD1	2.60 (8)	0.06	0.04	0.08	1.01	0.59	2.29
HD1*: Standard reference strain of <i>Bacillus thuringiensis</i>. LC₅₀ values represent the median lethal concentration required to cause 50% mortality of <i>Plutella xylostella</i> larvae. The 95% fiducial limits indicate the confidence interval for each LC₅₀ estimate. χ^2 represents the goodness-of-fit of the probit model, and df (degrees of freedom) = number of concentrations tested – 1.							

Discussion

The present study provided a comprehensive characterization of indigenous *B. thuringiensis* isolates through their morphological traits, crystal structures, toxin gene profiles, protein banding patterns and biological activity against *P. xylostella*, a globally important crucifer pest (Paudel et al. 2022; Zalucki et al. 2012). The combined evidence from each analytical approach clearly demonstrated that these isolates exhibit substantial diversity, which has significant implications for their potential use in developing novel Bt-based biopesticides.

Variation in colony morphology observed among the isolates is consistent with earlier reports describing the inherent heterogeneity present within Bt populations (Rabha et al. 2017). Although colony morphology is not a direct indicator of toxicity, it often reflects underlying genetic and physiological

differences. The majority of isolates exhibited typical Bt-like characteristics, including off-white pigmentation and a “fried-egg” colony appearance. However, the presence of distinct colony morphologies suggests ecological adaptation and underlying genotypic variability within the populations. Consistent with our findings, earlier studies have reported that Bt colonies are typically white to off-white in color, with irregular margins and flat to slightly raised elevations (Mane et al. 2015; Padole et al. 2017).

Crystal morphology observed under scanning electron microscopy further substantiated this. The occurrence of bipyramidal and cuboidal crystals corresponded well with Cry1 and Cry2 toxin families, respectively as supported by PCR and sequence data (Crickmore et al. 2016). Bipyramidal crystals, commonly associated with Cry1 toxins (e.g., *cry1Aa*, *cry1Ac*) (Al-Thani et al. 2022), predominated across isolates, aligning with widespread detection of *cry1* genes. In contrast, cuboidal crystals indicated the presence of Cry2 toxins (e.g., *cry2Ab*) (Adalat et al. 2017; Boonmee et al. 2019) in specific isolates. This variation reflects the coexistence of multiple Cry toxin types across isolates, which is advantageous for pest management, as toxin diversity broadens insecticidal spectra and contributes to resistance delay. Cry and Vip proteins differ in their structure, receptor binding, and mode of action within the insect midgut, thereby minimizing the risk of cross-resistance by Jurat-Fuentes and Crickmore (2017). Notably, Vip3 proteins bind to distinct midgut receptors compared to Cry toxins, and accumulating evidence indicates limited cross-resistance between these toxin classes in lepidopteran pests (Yang et al. 2021).

The combined use of Cry and Vip proteins is widely considered as an effective strategy for delaying resistance evolution, as these toxins act through independent modes of actions. According to Figueiredo et al. (2019), combinations of Vip3 and Cry1 proteins exhibited greater toxicity than Vip3 and Cry2 combinations, leading to significantly enhanced efficacy against *Spodoptera frugiperda* (Smith). This enhanced toxicity may be attributed to complementary interactions between toxin binding sites and midgut receptors, resulting in improved insecticidal performance. Pyramiding toxins with different mechanisms has been shown to enhance durability of control measures and delay resistance development in target insect populations (Tabashnik and Carrière 2017; Carrière et al. 2020). Moreover, Vip3 proteins have been shown to remain effective against insect populations resistant to Cry toxins, highlighting their importance in integrated resistance management and the development of next-generation biopesticides (Yang et al. 2021). Studies on *Mythimna separata* (Walker) have revealed substantial variability in susceptibility to Bt toxins, with LC_{50} values ranging from 1.6 to 78.6 $\mu\text{g/g}$, and Vip3 proteins often exhibiting higher potency than several Cry toxins. Additionally, combinations of Cry and Vip toxins showed significant synergistic factors ranging from 2.2 to 9.2 resulting in enhanced mortality (Yang et al. 2018). Collectively, these findings support the potential of Cry/Vip toxin combinations to improve insecticidal efficacy and delay resistance development in *P. xylostella*.

Consequently, isolates exhibiting diverse crystal morphologies and corresponding toxin profiles represent promising candidates for sustainable pest management. In the present study, the predominance of bipyramidal crystals across isolates was consistent with the widespread detection of *cry1* genes, while cuboidal crystal structures observed in a subset of isolates corresponded with the

presence of *cry2* genes. In contrast, although *vip3* genes were detected through PCR analysis, their presence could not be inferred from crystal morphology, as Vip3 proteins are secreted during the vegetative growth phase and do not form parasporal inclusions. Overall, the observed crystal diversity was in strong agreement with the distribution of Cry toxin genes identified through molecular characterization.

SDS–PAGE profiling showed multiple distinct protein bands, with major bands at ~ 124 kDa representing Cry1 protoxins, and additional bands around ~ 90 kDa and ~ 65 kDa corresponding to Vip3A and Cry2 proteins, respectively. These findings agree consistent with previous studies reporting the coexistence of Cry and Vip proteins in diverse Bt isolates (Fun et al. 2016; Gothandaraman et al. 2022). The presence of multiple toxins within a single isolate is particularly significant, as synergistic interactions between Cry1, Cry2 and Vip3A proteins have been shown to enhance insecticidal potency and reduce the likelihood of resistance development in lepidopteran pests (Bravo et al. 2015; Slamti et al. 2026). PCR amplification further confirmed the genetic diversity of the isolates, with all twelve isolates carrying *cry1*, while *cry2* and *vip3A* were present in varying combinations. Similar patterns of gene distribution have been observed among Indian Bt isolates (Navya et al. 2021), indicating that certain gene families particularly *cry1* are widespread, whereas other toxin genes occur less frequently. The absence of *cry9* in all isolates suggests either lower ecological prevalence of this gene family in the sampled environment or that these isolates are predominantly Cry1 and Vip3 producing types, which aligns with the observed crystal and protein profiles.

Toxicity bioassays against *P. xylostella* revealed significant variability in virulence among the isolates, as evidenced by dose-dependent mortality and a wide range of LC₅₀ values. Similar variability in susceptibility to *Bacillus thuringiensis* has been reported in other lepidopteran pests; for instance, bioassays against *Euprosterna elaeasa* showed LC₅₀ values ranging from 0.84 to 1.25 mg mL⁻¹ across different Bt isolates, including ABTS-1857 var. *aizawai*, GC-91 var. *aizawai*, HD1 var. *kurstaki*, and SA-12 var. *kurstaki* (Plata-Rueda et al. 2020). Notably, isolates RM12, T20B and T32 exhibited LC₅₀ values comparable to the reference strain HD1, which is widely used in commercial Bt formulations (George and Crickmore 2012). The high bioefficacy of these isolates can likely be attributed to the presence of multiple toxin gene families and the expression of multiple insecticidal proteins, as evident from both PCR and SDS–PAGE analyses. The probit regression models showed a good fit to the dose–response data, as indicated by non-significant chi-square values across all isolates. Such goodness of fit validates the accuracy of the estimated LC₅₀ and LC₉₅ values and confirms the reliability of the bioassay procedure used. Furthermore, the statistical interpretation, based on overlap of fiducial limits, rather than simple numerical comparison, ensured a scientifically robust and unbiased evaluation of the toxicity among the isolates, as recommended in probit-based toxicity assessments (Postelnicu 2025; Navik et al. 2019).

Despite these promising findings, certain limitations of the present study should be acknowledged. Although insecticidal activity was primarily evaluated under controlled laboratory conditions, bioefficacy of *B. thuringiensis* isolates using spore–crystal mixtures and culture broth was also assessed under greenhouse conditions. However, comprehensive field validation across diverse agro-ecological

conditions was not conducted. In addition, the genetic stability of the identified toxin genes was not examined over successive generations. Furthermore, while multiple toxin genes were detected within individual isolates, their potential synergistic interactions among Cry and Vip proteins were not experimentally evaluated. Future studies should therefore focus on long-term stability assessments, detailed interaction studies, and large-scale field trials to validate the efficacy, consistency and durability of these isolates under practical conditions.

Conclusions

The identification of highly potent indigenous isolates is especially important in the context of increasing resistance of *P. xylostella* to synthetic insecticides and reports reduced susceptibility to certain Bt formulations in field populations. The isolates RM12, T20B and T32, which exhibited toxicity comparable to HD1, demonstrate strong potential for development into microbial biopesticides tailored to regional pest management needs. Their multi-toxin profiles may enhance field efficacy and facilitate their integration into resistance management programs aimed at prolonging the durability of Bt-based control strategies.

Overall, this study provides a comprehensive characterization of indigenous *B. thuringiensis* isolates, and identifies novel, highly potent strains for effective management of *P. xylostella*. However, further validation through field-level evaluations, formulation optimization, and long-term efficacy assessments under diverse agroecological conditions is essential to confirm their practical applicability and scalability. Additionally, future studies involving whole genome sequencing will provide deeper insights into the complete toxin gene repertoire of these isolates.

Declarations

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Author Contribution

R.N.S.N. conducted the experiments as part of her doctoral research. V.B. conceptualized and designed the study. R.N.S.N., V.B., and L.R.C. prepared the manuscript. V.B. and M.R. provided research materials, assisted with the experimental work, and performed data analysis. M.R., M.M., and L.R.C. reviewed and edited the manuscript. All authors reviewed the manuscript.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

1. Afzal MB, Ijaz M, Abbas N, Shad SA, Serrão JE (2024) Resistance of lepidopteran pests to *Bacillus thuringiensis* toxins: evidence of field and laboratory evolved resistance and cross-resistance, mode of resistance inheritance, fitness costs, mechanisms involved and management options. *Toxins* 12;16(7):315.
2. Adalat R, Saleem F, Crickmore N, Naz S, Shakoory AR (2017) In vivo crystallization of three-domain Cry toxins. *Toxins* 9(3):80.
3. Altschul, SF, W Gish, W Miller, EW Myers, and DJ Lipman (1990) Basic local alignment search tool. *J Mol Biol* 215(3):403-410.
4. Al-Thani A, Ashfaq MY, Al-Thani R, Hassan ZU, Jaoua S (2022) Application of scanning electron microscopy and MALDI-TOF MS in the exploration of the parasporal insecticidal crystals of *Bacillus thuringiensis* strains isolated from Qatar soil. *Bioresour Technol Rep.* 19:101134.
5. Aswathi N, Balakrishnan N, Srinivasan T, Kokiladevi E, Raghu R (2024) Diversity of Bt toxins and their utility in pest management. *Egypt J Biol Pest Control* 34(1):40.
6. Bel Y, Ferré J, Hernández-Martínez P (2020) *Bacillus thuringiensis* toxins: functional characterization and mechanism of action. *Toxins* 12(12):785
7. Bermúdez NC, Pava ND, Nascimento DV, Ribeiro LM, Siqueira HA, Torres JB (2024) Fitness costs in diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) resistant to lufenuron, a chitin-synthesis inhibitor insecticide. *Insects* 2;15(11):856.
8. Bhardwaj K, Singh S. Biofabricated nanoparticles: Their delivery and utility in *Plutella xylostella* management (2022) *Indian J Biochem Biophys* 59(4):399-404.
9. Boonmee, K, SN-R Thammasittirong, and A Thammasittirong (2019) Molecular characterization of lepidopteran-specific toxin genes in *Bacillus thuringiensis* strains from Thailand 3 *Biotech* 9(4):1-11.
10. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal biochem* 72(1-2):248-54.
11. Bravo A, Martínez de Castro D, Sánchez J, Cantón PE, Mendoza G, Gómez I, Pacheco S, García-Gómez BI, Onofre J, Ocelotl J (2015) Mechanism of action of *Bacillus thuringiensis* insecticidal toxins and their use in the control of insect pests. *Comprehensive Sourcebook of Bacterial Protein Toxins* 4:858-873.

12. Carrière Y, Brown ZS, Downes SJ, Gujar G, Epstein G, Omoto C, Storer NP, Mota-Sanchez D, Søgaaard Jørgensen P, Carroll SP (2020) Governing evolution: a socioecological comparison of resistance management for insecticidal transgenic Bt crops among four countries. *Ambio* 49(1):1-6.
13. Crickmore N (2016) *Bacillus thuringiensis* resistance in *Plutella*—too many trees?. *Curr Opin Insect Sci* 15:84-8
14. Crickmore N, Berry C, Panneerselvam S, Mishra R, Connor TR, Bonning BC (2021) A structure-based nomenclature for *Bacillus thuringiensis* and other bacteria-derived pesticidal proteins. *J Invertebr pathol* 186:107438.
15. Eslamloo G, Ito T (2025) Insecticidal efficacy of non-Bt bacterial strains against diamondback moth, *Plutella xylostella* (L.). *Front Sustain Food Syst* 9:1591114.
16. Figueiredo CS, Lemes ARN, Sebastiao I, Desidério JA (2019) Synergism of the *Bacillus thuringiensis* Cry1, Cry2, and Vip3 proteins in *Spodoptera frugiperda* control. *Appl Biochem Biotechnol* 188(3):798-809.
17. Frolov AN, Zakharova YA (2025) Highly Improved Captures of the Diamondback Moth, *Plutella xylostella*, Using Bimodal Traps. *Insects* 16(9):881.
18. Fun, CP, X Rathinam, AH Ghazali, and S Subramaniam (2016) Characterization of a native *Bacillus thuringiensis* isolates from Malaysia that produces exosporium-enclosed parasporal inclusion. *Emir J Food Agric* 28(9):653-659.
19. Aaron J. Gassmann, Dominic D. Reisig (2023) Management of Insect Pests with Bt Crops in the United States. *Annu Rev Entomol* 68:31-49. <https://doi.org/10.1146/annurev-ento-120220-105502>
20. George Z, Crickmore N (2012) *Bacillus thuringiensis* applications in agriculture. In: Sansinenea E (ed) *Bacillus thuringiensis* biotechnology, 1st edn. Springer, Dordrecht.
21. Gonzalez-Vazquez MC, Vela-Sanchez RA, Rojas-Ruiz NE, Carabarin-Lima A (2021) Importance of Cry proteins in biotechnology: Initially a bioinsecticide, now a vaccine adjuvant. *Life* 11(10):999.
22. Gothandaraman R, Venkatasamy B, Thangavel T, Eswaran K, Subbarayalu M (2022) Molecular characterization and toxicity evaluation of indigenous *Bacillus thuringiensis* isolates against key lepidopteran insect pests. *Egypt J Biol Pest Control* 32(1):143.
23. Gupta M, Kumar H, Kaur S (2021) Vegetative insecticidal protein (Vip): a potential contender from *Bacillus thuringiensis* for efficient management of various detrimental agricultural pests. *Front microbiol* 12:659736.
24. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
25. Jurat-Fuentes JL, Crickmore N (2017) Specificity determinants for Cry insecticidal proteins: Insights from their mode of action. *J Invertebrate Pathol* 142:5-10.
26. Jurat-Fuentes JL, Heckel DG, Ferré J (2021) Mechanisms of resistance to insecticidal proteins from *Bacillus thuringiensis*. *Annu Rev Entomol* 66:121-40.
- Kashyap U, Garg S, Arora P (2024) Pesticide pollution in India: environmental and health risks, and policy challenges. *Toxicol Rep* 13:101801.

27. Kashyap U, Garg S, Arora P. Pesticide pollution in India: environmental and health risks, and policy challenges (2024) *Toxicol Rep* 1;13:101801.
28. Kaur M, Thakur M, Sharma KC (2021) Biological and life table parameters of diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Yponomeutidae) from five different geographical regions of India. *Phytoparasitica* 49(5):819-27.
29. Kumar A, Choudhary JK, Singh TK, Singh R, Shukla M, Singh S, Singh VK, Tripathi SK (2025) Efficacy of novel insecticides and bio-pesticides for diamond back moth (*Plutella xylostella*) management in cabbage. *Veg Sci* 52(01):191-5.
30. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685. <https://doi.org/10.1038/227680a0>
31. Li Z, Zalucki MP, Yonow T, Kriticos DJ, Bao H, Chen H, Hu Z, Feng X, Furlong MJ (2016) Population dynamics and management of diamondback moth (*Plutella xylostella*) in China: the relative contributions of climate, natural enemies and cropping patterns. *Bull Entomol Res* 106(02):197–214. <https://doi.org/10.1017/s0007485315001017>
32. Mane V, Shinde H, Pagar T, Garud M (2015) Characterization and Screening of *Bacillus thuringiensis* Isolated from Nashik and Ahmednagar Region of Maharashtra. *Int J Res Agric Sci* 2(4):198-201.
33. Navik O, Ramya RS, Varshney R, Jalali SK, Shivalingaswamy TM, Rangeshwaran R, Lalitha Y, Patil J, Ballal CR (2019) Integrating biocontrol agents with farmer's practice: impact on diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) and cabbage yield. *Egypt J Biol Pest Control* 29(1):1-7. <https://doi.org/10.1186/s41938-019-0140-x>
34. Navya R, Balasubramani V, Raveendran M, Murugan M, Lakshmanan A (2021) Diversity of indigenous *Bacillus thuringiensis* isolates toxic to the diamondback moth, *Plutella xylostella* (L.) (Plutellidae: Lepidoptera). *Egypt J Biol Pest Control* 31(1):1–7. <https://doi.org/10.1186/s41938-021-00495-2>
35. Navya RN, Balasubramani V, Raveendran M, Murugan M, Lakshmanan A. Characterization of indigenous *Bacillus thuringiensis* isolate RM11 toxic to the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). *Egyptian Journal of Biological Pest Control*. 2022 May 16;32(1):56.
36. Padole DA, Moharil MP, Ingle KP, Munje S (2017) Isolation and characterization of native isolates of *Bacillus thuringiensis* from Vidarbha Region. *Int J Curr Microbiol Appl Sci* 6:798-806. <https://doi.org/10.20546/ijcmas.2017.601.094>
37. Pasupathi E, Edward YS, Kannan M, Ramalingam J (2022) Monitoring the development of resistance in diamondback moth (DBM) I (L.) to diamide group of insecticides. *J Entomol Res* 46(1):111-5.
38. Paudel A, Yadav PK, Karna P (2022) Diamondback moth *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae); a real menace to crucifers and its integrated management tactics. *Turk. J. Agric. Food Sci.* 10(12):2504-15.
39. Plata-Rueda A, Quintero HA, Serrão JE, Martínez LC (2020) Insecticidal activity of *Bacillus thuringiensis* strains on the nettle caterpillar, *Euprosterina elaeasa* (Lepidoptera: Limacodidae). *Insects* 11(5):310.

40. Postelnicu T (2025) Probit analysis. In International encyclopedia of statistical science 2025 (pp. 1991-1994). Berlin, Heidelberg: Springer Berlin Heidelberg.
41. Punniyakotti P, Vinayagam S, Rajamohan R, Priya SD, Moovendhan M, Sundaram T (2024) Environmental fate and ecotoxicological behaviour of pesticides and insecticides in non-target environments: Nanotechnology-based mitigation strategies. J Environ Chem Eng 12(5):113349.
42. Qu YJ, Chen JC, Li HJ, Wang SY, Zhang XY, Hoffmann AA, Wang SS, Wei SJ (2026) Temporal dynamics of insecticide-resistance mutations in the diamondback moth *Plutella xylostella* (Linnaeus, 1758) within northern immigration regions. J of Asia Pac Entomol 29(1):102532.
43. Rabha M, Sharma S, Acharjee S, Sarmah BK (2017) Isolation and characterization of *Bacillus thuringiensis* strains native to Assam soil of North East India. 3 Biotech 7(5):303.
44. Ramalakshmi A, Udayasuriyan V (2010) Diversity of *Bacillus thuringiensis* isolated from Western Ghats of Tamil Nadu State, India. Curr Microbiol 61:13-18. <https://doi.org/10.1007/s00284-009-9569-6>
45. Sahin B, Gomis-Cebolla J, Güneş H, Ferre J (2018) Characterization of *Bacillus thuringiensis* isolates by their insecticidal activity and their production of Cry and Vip3 proteins. PLoS one 13(11):e0206813.
46. Sambrook J, Russell DW (2001) Preparation and analysis of eukaryotic genomic DNA. In: Argentine J (ed) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York, pp 547–576.
47. Slamti L, Lereclus D (2026) *Bacillus thuringiensis* and insects: a century of intimate history. J Bacteriol :e00381-25.
48. Sneath PH, Mair NS, Sharp ME, Holt JG. Bergey's manual of systematic bacteriology. 1986. Williams and Wilkins. Co. Baltimore.
49. Sun D, Wang M, Guo L, Shentu X, Yu X, Crickmore N, Zhou X, Zhang Y, Guo Z (2025) Reverse engineering high-level resistance to Bt Cry1Ac toxin in *Plutella xylostella* reveals a hormonal regulatory feedback pathway. Pestic Biochem Physiol 210:106382.
50. Syed T, Askari M, Meng Z, Li Y, Abid MA, Wei Y, Guo S, Liang C, Zhang R (2020) Current insights on vegetative insecticidal proteins (Vip) as next generation pest killers. Toxins 12(8):522.
51. Tabashnik BE, Carrière Y (2017) Surge in insect resistance to transgenic crops and prospects for sustainability. Nat biotechnol 35(10):926-35.
52. Tamilselvan R, Kennedy JS, Suganthi A (2021) Monitoring the resistance and baseline susceptibility of *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) against spinetoram in Tamil Nadu, India. Crop protection 142:105491.
53. Xie J, He W, Qiu M, Lin J, Shu H, Wang J, Liu L (2026) Field-Evolved Resistance to Bt Cry Toxins in Lepidopteran Pests: Insights into Multilayered Regulatory Mechanisms and Next-Generation Management Strategies. Toxins 18(2):60.
54. Xiong L, Liu Z, Shen L, Xie C, Ye M, Li Z, Zhang Z, Li J, Dong Y, You M, You S (2021) A novel reference for Bt-resistance mechanism in *Plutella xylostella* based on analysis of the midgut transcriptomes.

55. Xu J (2025) Laboratory Rearing Methods and Observations of *Plutella xylostella* L. Under Controlled Conditions. Mol Entomol 23:16.
56. Yang F, Kerns DL, Little NS, Santiago González JC, Tabashnik BE (2021) Early warning of resistance to Bt toxin Vip3Aa in *Helicoverpa zea*. Toxins 13(9):618
57. Zhou L, Wang Y, Liu ZY, Liu X, Zhai Z, Cao SK, Zhao QQ, Zaghloul HA, Shi XB, Yu H, Su H. (2025) Biological activity and field efficacy of *Bacillus thuringiensis kurstaki* strains with protein film adjuvants (PFAs) against *Plutella xylostella* (Lepidoptera: Plutellidae). Pest Manag Sci (7):4108-20.

Figures

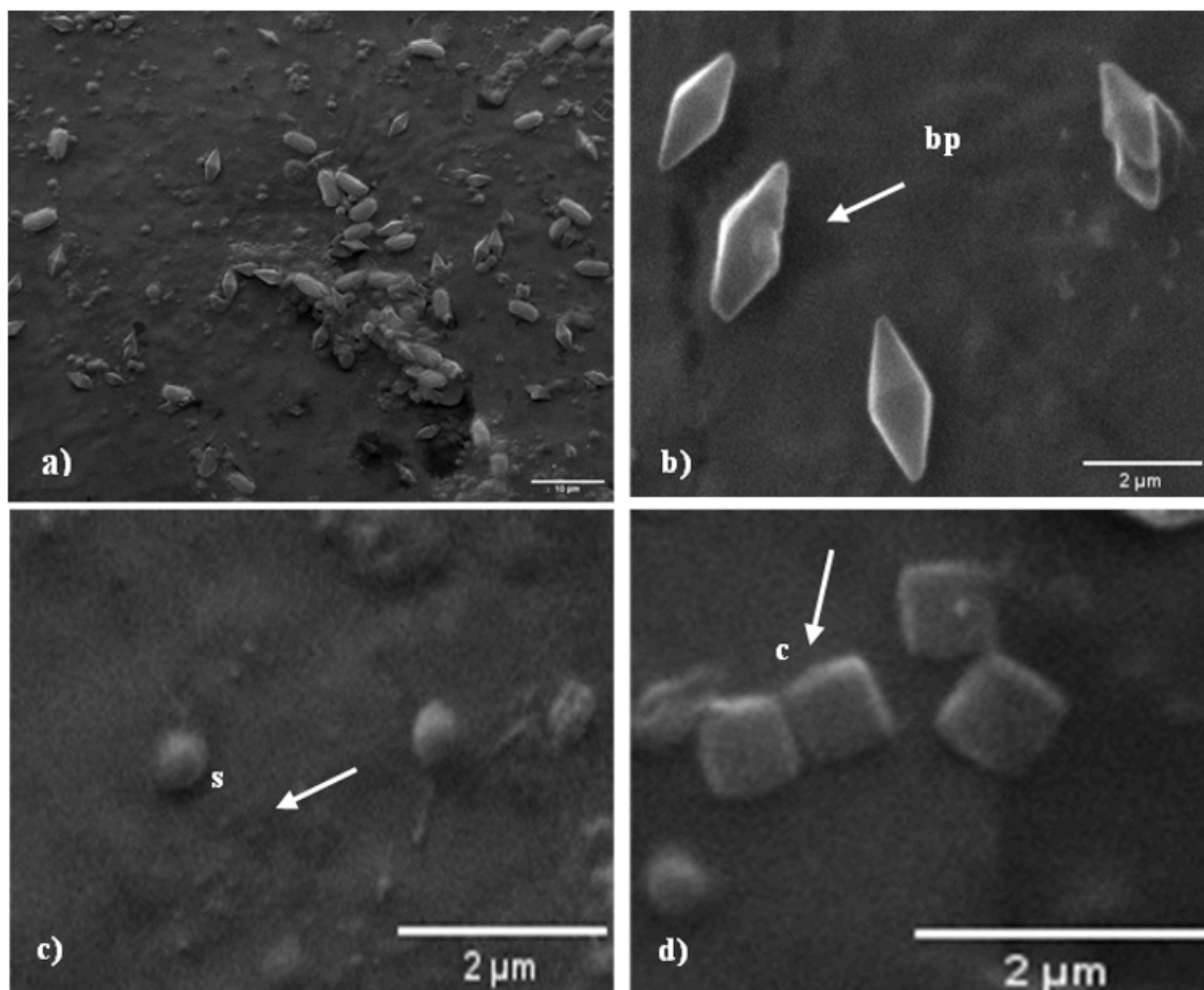


Figure 1

Scanning electron microscopy images of parasporal crystal inclusions produced by indigenous *Bacillus thuringiensis* isolates. (A) Representative SEM image showing crystal morphologies (B) Bipyramidal

(bp) (C) Spherical (s) and (D) Cuboidal (c) crystal structures observed among the reference strain HD1 and isolates RM12, RM17, RM22, RM29, T15, T20B, T29, T32, T152, T191, T380 and T405.

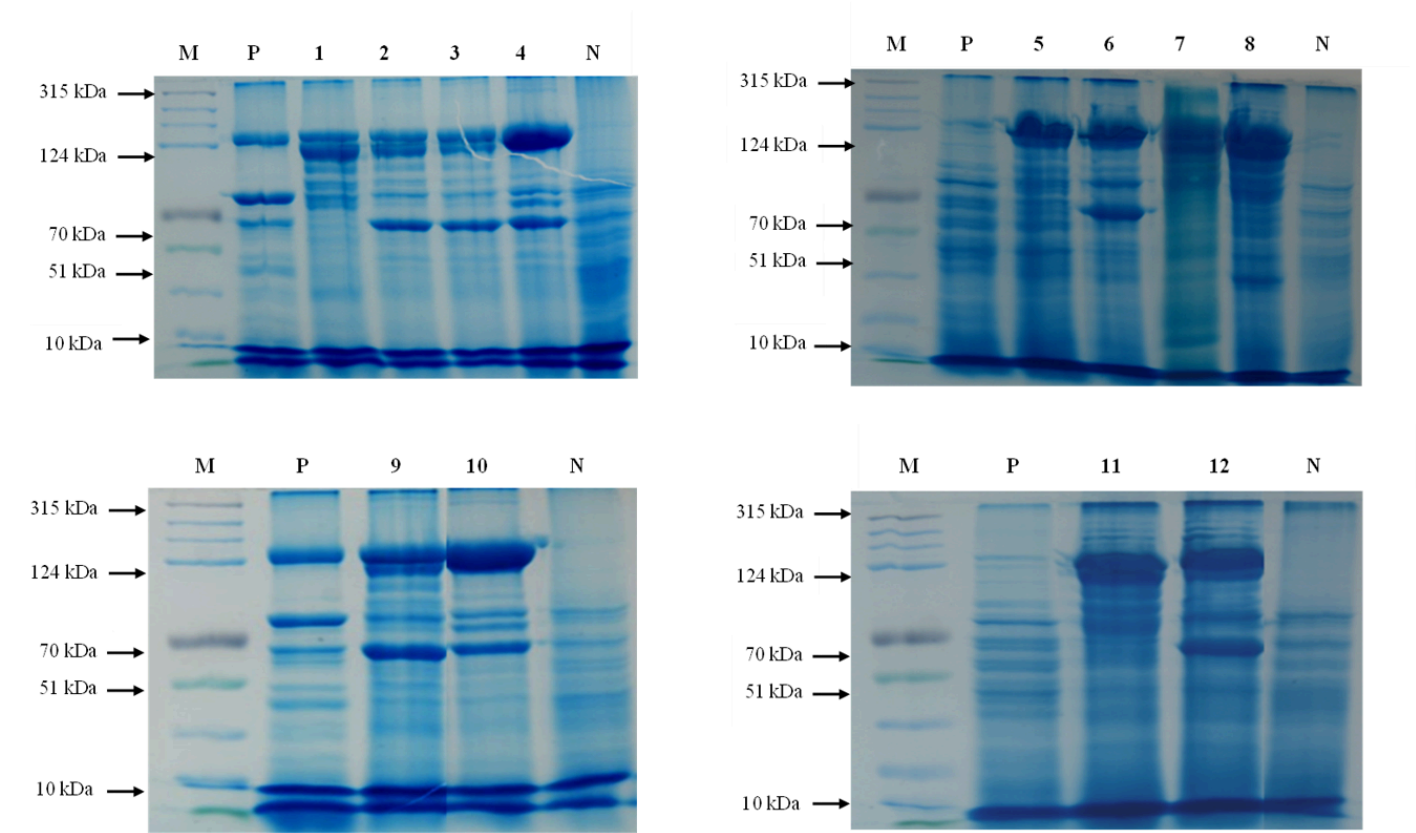


Figure 2

Protein profiling of *Bacillus thuringiensis* isolates by SDS-PAGE showing major protein bands corresponding to Cry1 (~124 kDa), Cry2 (~65 kDa) and Vip3 (~90 kDa). Lane M - Broad range marker, Lane 1 to 12 – T20, T380, T29, T405, RM12, RM27, RM29, RM17, T15,T32, T152, RM22.

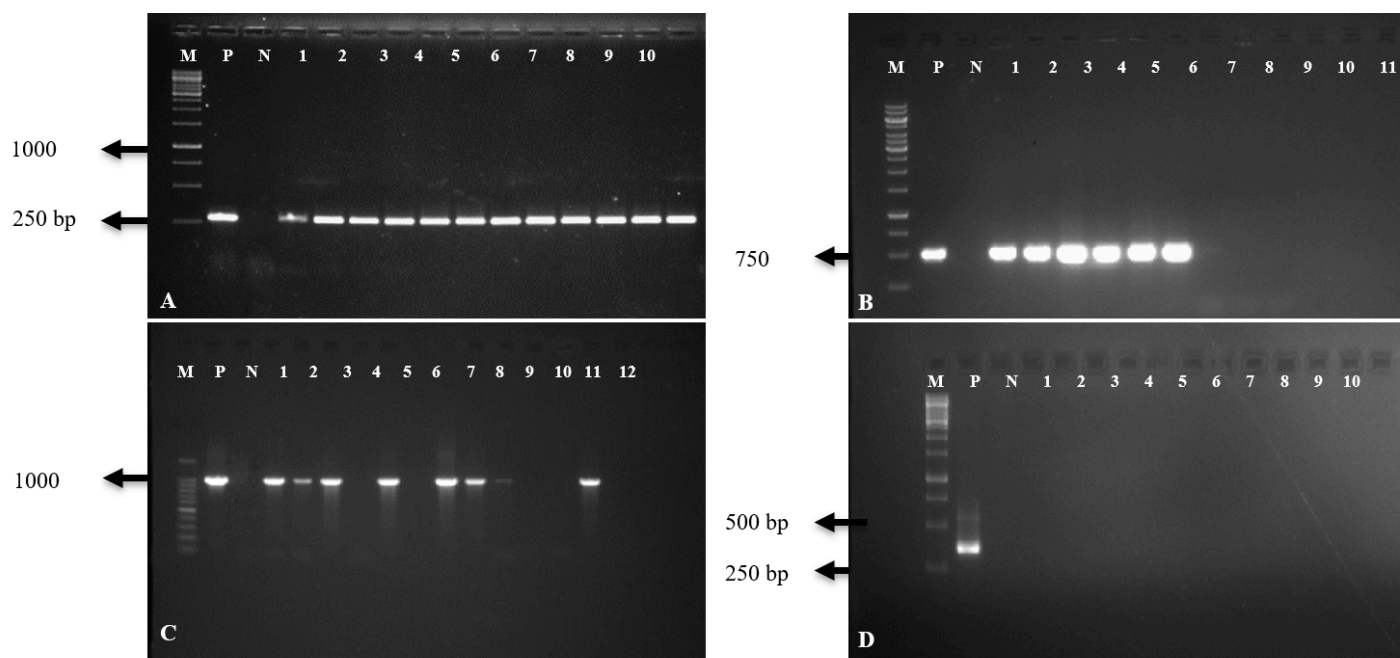


Figure 3

PCR screening of indigenous *Bacillus thuringiensis* isolates for detection of toxin genes A) *cry1* gene B) *cry2* gene C) *cry9* gene and D) *vip3* gene

Lanes: M – molecular marker (1 kb DNA ladder for panels A, B, and D; 100 bp DNA ladder for panel C); P – positive control (*B. thuringiensis* subsp. *kurstaki* HD1); N – negative control (acrystalliferous strain 78/11). Panels A, C, and D: Lane 1 – RM12; Lane 2 – RM17; Lane 3 – RM22; Lane 4 – RM29; Lane 5 – T15; Lane 6 – T20B; Lane 7 – T29; Lane 8 – T32; Lane 9 – T152; Lane 10 – T191; Lane 11 – T380; Lane 12 – T405; Panel B: Lane 1 – RM22; Lane 2 – T15; Lane 3 – T29; Lane 4 – T32; Lane 5 – T380; Lane 6 – T405; Lane 7 – RM12; Lane 8 – RM17; Lane 9 – RM29; Lane 10 – T20B; Lane 11 – T152; Lane 12 – T191.

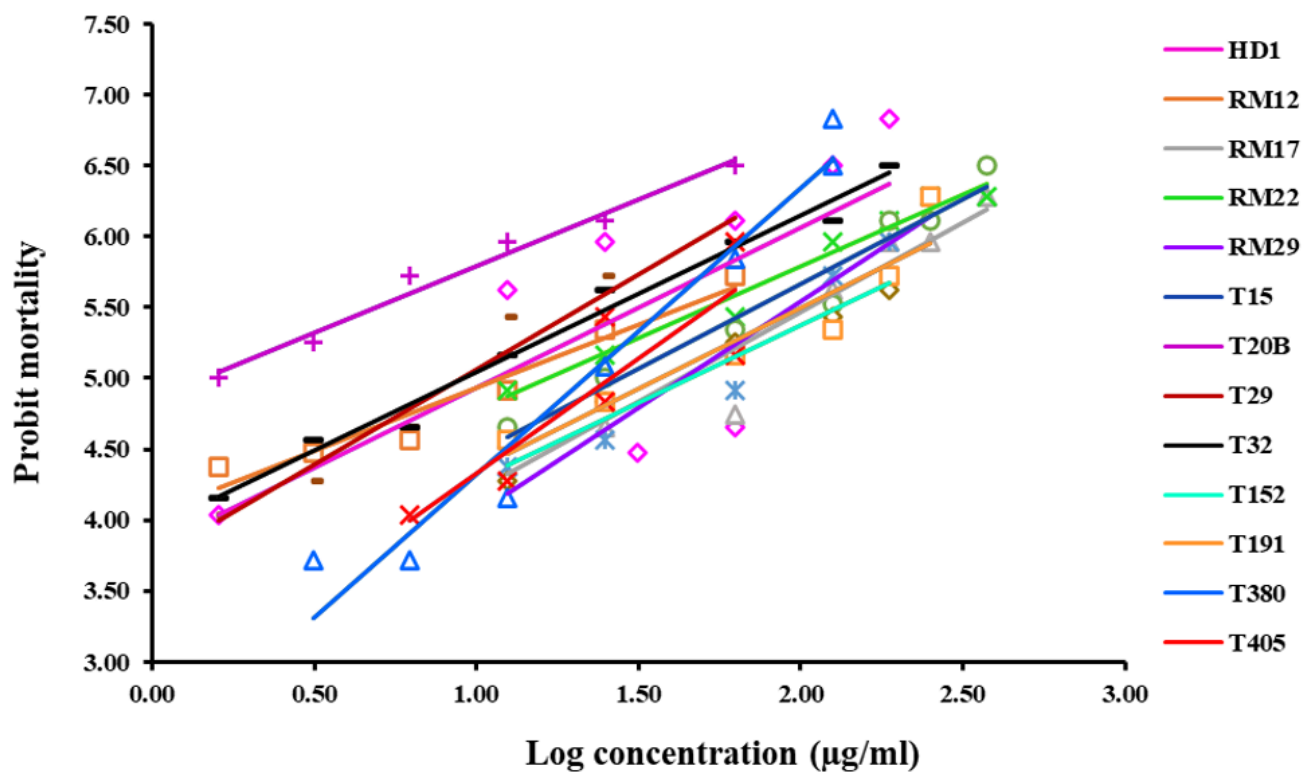


Figure 4

Probit analysis of indigenous *Bacillus thuringiensis* (Bt) isolates RM12, RM17, RM22, RM29, T15, T20B, T29, T32, T152, T191, T380, and T405, along with the reference strain HD1, harboring *cry1* genes. Probit mortality is plotted against log-transformed concentrations ($\mu\text{g ml}^{-1}$) to establish dose-response relationships. The regression lines indicate variation in toxicity among isolates

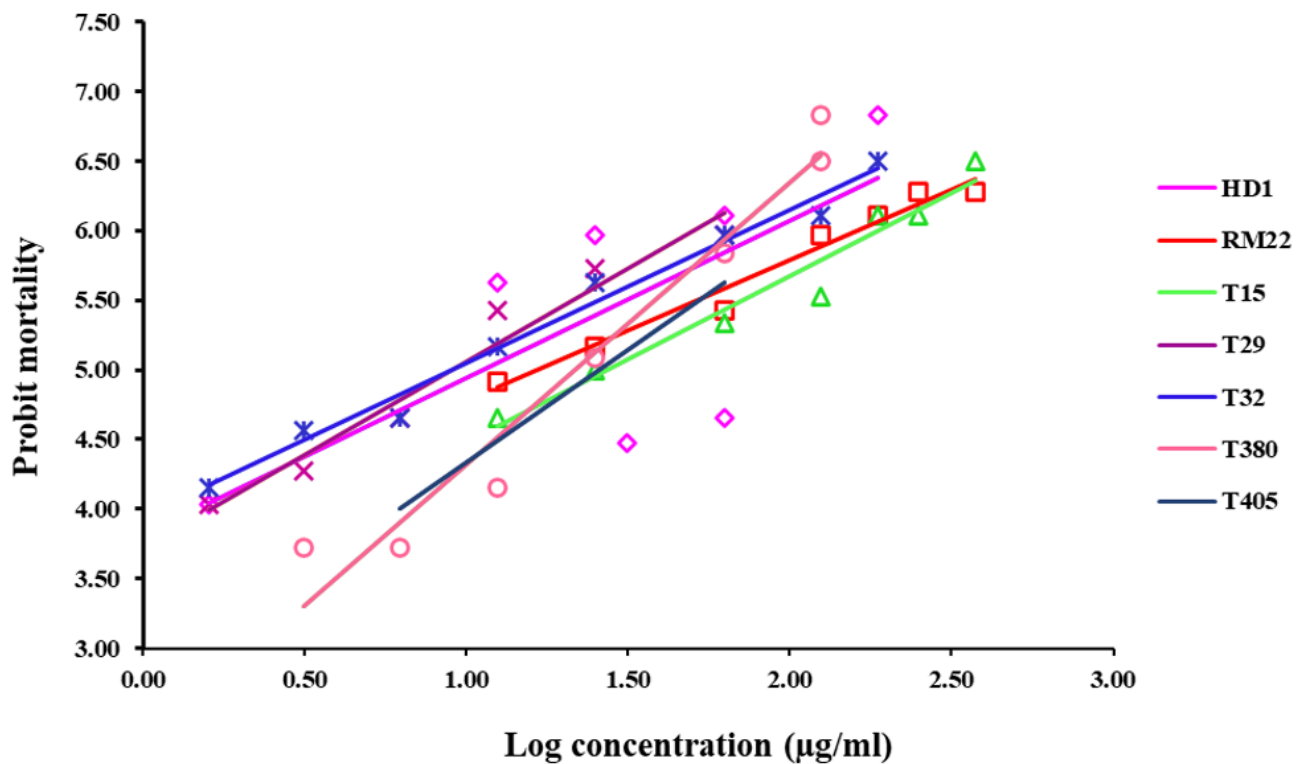


Figure 5

Probit analysis of indigenous *Bacillus thuringiensis* (Bt) isolates RM22, T15, T29, T32, T380, and T405 along with the reference strain HD1, harboring *cry2* genes. Probit mortality is plotted against log-transformed concentrations ($\mu\text{g ml}^{-1}$) to establish dose-response relationships. The regression lines indicate variability in toxicity among isolates.

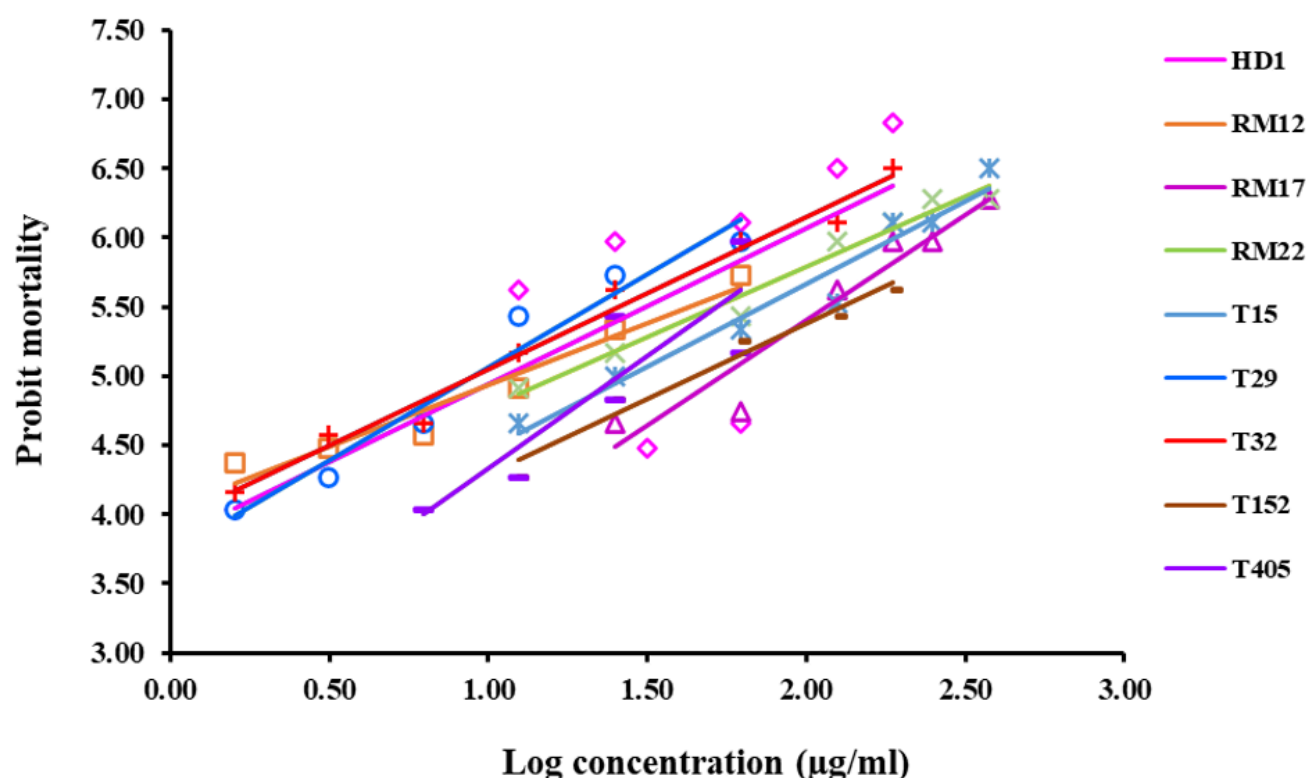


Figure 6

Probit analysis of indigenous *Bacillus thuringiensis* (Bt) isolates RM12, RM17, RM22, T15, T29, T32, T152, and T405, along with the reference strain HD1, harboring *vip3A* genes. Probit mortality is plotted against log-transformed concentrations ($\mu\text{g ml}^{-1}$) to describe dose-response relationships. The regression lines reflect differences in insecticidal activity among isolates.

Supplementary Files

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- [supplementaryfile.docx](#)