Characterization and Evaluation of Native *Bacillus thuringiensis* Isolate T121 Toxic to *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae)[1]

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Abstract The present study focused on the molecular characterization and toxicity analysis of the *Bacillus thuringiensis* Berliner (Bt) isolate T121 against *Henosepilachna vigintioctopunctata* (F.) (Coleoptera: Coccinellidae). Colonies of T121 growing on solid media were circular, with creamy white hues and raised centers. Scanning electron microscopy revealed the presence of cuboidal and spherical crystals in liquid media. Polymerase chain reaction screening confirmed the presence of binary toxin *vip1* and *vip2* in addition to *cry3Aa* gene. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed four prominent bands at 74, 65, 48, and 31 kDa. In toxicity assays, T121 exhibited a median lethal concentration of 4.675 μg/ml against *H. vigintioctopunctata*. These findings demonstrate that the Bt isolate T121 offers promising prospects for development as a component of a sustainable pest management strategy against *H. vigintioctopunctata* and perhaps other coleopteran pests.

Key Words *Bacillus thuringiensis*, Bt isolate T121, SDS-PAGE, LC_{50}, *Henosepilachna vigintioctopunctata*

The immature and adult beetles in the subfamily Epilachninae (Coleoptera: Coccinellidae) are highly destructive pests of crops in the Solanaceae (Rajagopal and Trivedi 1989), Cucurbitaceae (Al-Digail et al. 2012, Hossain et al. 2009, Tayde and Simon 2013, Uikey et al. 2016), Compositae (Katakura 1997), and Fabaceae and Malvaceae (Jamwal et al. 2013). These beetles, particularly *Henosepilachna vigintioctopunctata* (F.) and *Epilachna dodecastigma* (Wiedemann), have reportedly reduced crop yields by as much as 80% in India (Haldhar et al. 2018). Growers

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have relied on various chemical pesticides as the primary means to manage these pests due to their immediate knockdown effect (Khursheed and Raj 2013, Nayak et al. 2022). Nevertheless, chemical pesticide-based management methods have drawbacks, including impacting nontarget organisms, resurgence of secondary pests, and development of pesticide resistance in insect populations. In the search for sustainable and ecofriendly pest management tactics, the use of *Bacillus thuringiensis* Berliner (Bt) is receiving attention as a potential alternative option.

Bt belongs to the *Bacillus cereus* group whose members are rod-shaped, gram-positive, facultatively anaerobic, and spore forming (Sanahuja et al. 2011). Bt differs from other bacteria within the *B. cereus* group owing to its capacity to generate crystalline inclusions upon sporulation. These crystalline inclusions are composed of two distinct proteins, namely, Cry and Cyt toxins (known as delta-endotoxins), which exhibit specific insecticidal activity toward different insects (Schnepf et al. 1998). Apart from delta-endotoxins produced during sporulation, the vegetative phase yields Vip and Sip toxins (de Maagd et al. 2003). The proteins that exhibit coleopteran toxicity include Cry3, Cry7, Cry8, Cry43, Gpp34 (Cry34)/Tpp35 (Cry35), Mpp23 (Cry23)/Xpp37 (Cry37), Mpp51 (Cry51), Mpp75 (Cry75), Vpb1 (Vip1)/Vpa2 (Vip2), Xpp22 (Cry22), and Xpp55 (Cry55) (Crickmore et al. 2021, Domínguez-Arrizabalaga et al. 2019). Within these protein families Cry3, Cry7, and Cry8 possess the capacity to affect a wide range of coleopteran species, implying that they might serve as a promising reservoir of toxins with unique insecticidal properties (Domínguez-Arrizabalaga et al. 2020). The aim of this study was to characterize the coleopteran toxin of the native Bt isolate T121 to determine its genetic diversity and its toxicity against *H. vigintioctopunctata.*

Materials and Methods

Bt strains. A native Bt isolate, T121, and the reference strain *B. thuringiensis* subsp. *tenebrionis* (Btt) were acquired from the Bt laboratory at the Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India. The T121 isolate was chosen from a pool of 50 Bt isolates following a preliminary screening bioassay using a spore crystal mixture at a concentration of 25 μg/ml (unpubl. data). The acrystalliferous strain 4Q7 was initially procured from the Bacillus Genetic Stock Centre, Columbus, OH, USA. These cultures were grown either in T3 broth at 30°C with vigorous agitation for 12 h in an orbital shaker (Orbitek, Scigenics, Chennai, India) or on T3 media agar plates at 30°C.

Bacterial colony morphology and crystal pattern. The morphology of the Bt colonies was examined visually after growth on T3 media agar plates. To examine spore crystal inclusions, 2 μl of a liquid culture of the Bt was evenly spread on glass slides, heat fixed, stained with Coomassie brilliant blue stain (G250), and examined under a bright-field microscope (Iscope, Euromex, The Netherlands) at 100× magnification. These cultures were established by inoculating 250 μl of a 12-h-old culture into 25 ml of T3 broth and then subjected to incubation at 30°C for 48 h with constant agitation at 200 rpm.

Spore crystal mixture isolation. After confirming the lysis of 90% of the cells, the broth culture was centrifuged at 4°C for 10 min at 7,000 rpm. The pellet

obtained was solubilized in 25 ml of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, and 1 mM phenylmethanesulfony fluoride [PMSF], pH 8.0). Subsequently, the pellet was subjected to a single wash with 25 ml of 0.5 M NaCl, followed by another two additional rinses with Tris-EDTA buffer. The resulting pellet of spore crystal was resuspended in 500 μl of nuclease-free water (Qiagen, Hilden, Germany) with the addition of 1 mM PMSF and stored at −20°C for further analysis.

Scanning electron microscopy (SEM). The spore crystal image was obtained using a Quanta 250 scanning electron microscope (FEI, Brno, Praha, Czech Republic) located at the Centre for Agricultural Nanotechnology, TNAU, Coimbatore, Tamil Nadu, India. For SEM, the spore crystal mixture was subjected to two washes with sterile nuclease-free water (Qiagen, Tegelen, The Netherlands). Subsequently, 100 μl of spore crystal mixture was added to 1,900 μl of sterile distilled water and subjected to sonication at 37 kHz to disperse aggregates and then vortexed. A volume of 30 μl from the resulting solution was applied to the SEM sample holder and left to dry. The sample was then coated with gold nanoparticles by using a sputter coating process, enabling the crystals and spores to acquire conductivity. The samples were placed in the vacuum chamber of SEM, and the imaging process was conducted at a voltage of 15 kV, with the sample positioned 4 mm away from the target.

Protein profiling with SDS-PAGE. The spore crystal mixture was resuspended in 1× sample solubilizing buffer (0.5 M Tris, pH 6.8; 0.1% bromophenol blue; 2% SDS; 5% β-mercaptoethanol; and 10% sucrose) before subjecting to incubation at 95°C for 10 min to facilitate protein denaturation. The denatured samples were analyzed on 12% SDS-PAGE following the protocol outlined by Laemmli (1970) by using 0.133% Coomassie brilliant blue R250 staining and destaining. The protein bands in the gel were compared with the prestained protein marker PGPMT2962 (Puregene, Genetix Biotech Asia Pvt Ltd., New Delhi, India) that spans a molecular weight range of 10–315 kDa.

Genomic DNA isolation and PCR sequence analysis. The genomic DNA was isolated from overnight-grown cultures following the methodology of Sambrook and Russell (2001). For PCR amplification, a quantity of 30–50 ng of the extracted DNA was used as a template. The PCR was performed using Nexus GX2 Eppendorf PCR master cycler. The reaction mixture consisted of 30–50 ng of total genomic DNA, forward and reverse primer at a 1 μM concentration each, 2× master mix (EmeraldAmp, Takara, Takara Bio India Pvt. Ltd., New Delhi, India), which contained deoxynucleoside triphosphates (dNTPs), Taq polymerase, and reaction buffer. The Vip1 primers were designed based on the conserved regions of *vip1Aa* (GU992203), *vip1Ad* (JQ855505), *vip1Bb* (HM485584; KR065727), *vip1Bc* (HM485583), and *vip1Ca* (KR065725) to characterize the *vip1* genes. The primers listed in Table 1 were used to perform PCR targeting *cry3*, *vip1*, and *vip2* genes. The PCR products obtained were examined through a 1.2% agarose gel. The PCR products were Sanger sequenced with corresponding gene-specific primers at Biokart India Pvt. Ltd., Bangalore, India. The gene sequences were analyzed using the BioEdit program (Hall 1999) and subjected to BLAST analysis against the nucleotide and protein databases. The resulting aligned sequences were submitted to GenBank to obtain accession numbers.

Insecticidal activity of Bt isolate T121 against *H. vigintioctopunctata*. A laboratory population of *H. vigintioctopunctata* was maintained from the field-

Table 1. Primer sequences used for amplification of *cry* and *vip* genes encoding coleopteran toxic Cry proteins.[a]

Target Gene	Sequences	Product Size (bp)	Annealing Temperature (°C)	Reference
cry3	F: 5′-CGTTATCGCAGAGAGATGACATTAAC-3′ R: 5′-CATCTGTTGTTTCTGGAGGCAAT-3′	589	52	Ben dov et al. (1997)
vip1	F: 5′-CAACAAGAATATCAKTCYATTC-3′ R: 5′-TTRATYGTAAMATTCATT-3′	~1,235	53	This study
vip2	F: 5′-GGATCCGATGAAAAGAATGGAGGG-3′ R: 5′-GTCGACTTAATTTGTTAATAATGTTG-3′	~1,390	57	Shi et al. (2004)

[a] F, forward; R, reverse.

collected adults and was consistently maintained in the laboratory (25 ± 1°C, 75 ± 5% relative humidity, and a photoperiod of 16:8 [light:dark] h). The bioassay was conducted using a spore crystal mixture of the Bt isolate T121 against first-instar grubs by using the leaf disc method. Freshly cut eggplant (*Solanum melongena* L.) leaves were used to cut circular discs 40 mm in diameter. Forty microliters of diluted spore crystal mixtures with concentrations ranging from 6.5 to 0.5 μg were spread on both sides of the leaf discs (20 μl/side) and air dried. Sterile distilled water was spread on leaf discs to serve as controls along with spore crystal suspensions from the standard strains Btt (positive control) and 4Q7 (negative control). Three replications were conducted per treatment, with 10 first-instar grubs per replicate. Mortality of the grubs was monitored at 24–72 h. Data were subjected to probit analysis (Finney 1971) using POLO-PC LeOra 2.0 software.

Results and Discussion

Colony and crystal morphology characterization. Bt has been documented to exhibit variations in color (white, off-white, and yellowish white) and type (resembling a fried egg or a glossy appearance). The texture may vary and can be described as circular, flat, or raised elevation, with complete or undulated margins (Abo-Bakr et al. 2020, Ma et al. 2023, Mukhija and Khanna 2018, Navya et al. 2021). The morphological characteristics of the Bt isolate T121, as well as the standard strains Btt and 4Q7, were examined by assessing various attributes such as color, elevation, margin, shape, and surface characteristics. All three Bt isolates exhibited a creamy white hue. The surface resembled a fried egg, circular with a raised elevation, and a complete margin.

Examination of the spore crystal mixture with bright-field microscopy and SEM revealed the presence of cuboidal and spherically shaped crystals in T121 (Fig. 1), whereas cuboidal-shaped crystals were observed with Btt. These results agree with descriptions by Naveenarani et al. (2022) and Soltani-Nezhad et al. (2022). Various

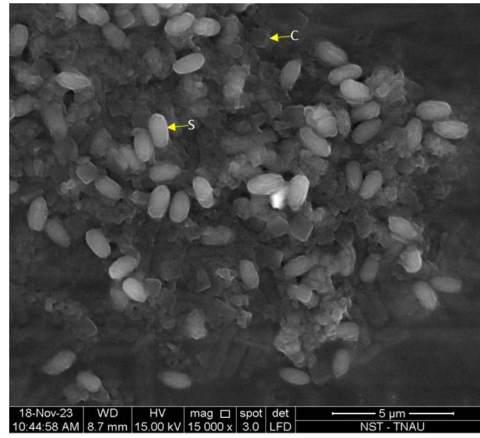

Fig. 1. Scanning electron micrograph of *Bacillus thuringiensis* isolate T121 (S, spores; C, cuboidal crystal).

shape types of crystals, including bipyramidal, rectangular, cuboidal, and spherical, are reportedly produced by Bt (Gothandaraman et al. 2022, Ramalakshmi and Udayasuriyan 2010).

PCR screening and DNA sequencing. PCR analysis confirmed the presence of binary toxin gene *vip1* and *vip2* in addition to *cry3* gene in the *Bt* isolate T121. Validation of the PCR products was achieved through Sanger sequencing using gene-specific primers. The acquired sequences were aligned, followed by Blastn analysis against National Center for Biotechnology Information (NCBI) nucleotide databases. This revealed 100% homology with the previously identified *cry* and *vip* genes available in the NCBI database. The nucleotide sequences of *cry3*, *vip1*, and *vip2* from the T121 Bt isolate were submitted to NCBI, and their GenBank accession numbers are OM777031, OR921178, and OR947062, respectively.

Protein profiling through SDS-PAGE. Examining the molecular weights of crystal proteins through SDS-PAGE profiling provides a valuable method for anticipating the existence of *cry* genes. The SDS-PAGE analysis of Bt isolate T121 revealed four prominent bands, with molecular weights of 74, 65, 48, and 31 kDa, as did the positive standard Btt (Fig. 2). The varied patterns of protein bands detected in Bt isolates suggest differences in biological activity and specificity against different insect pests. Nazarian et al. (2009) and Asokan et al. (2014) observed a range of electrophoretic patterns encompassing molecular weights ranging from 20 to 135 kDa. Our findings are consistent with the results reported by Rupar et al. (1991): they observed a prominent 74-kDa protein band in the EG2838 isolate and a 73-kDa protein in the EG2158 that also generated minor crystal proteins of approximately 31 and 29 kDa. Notably, these proteins exhibit

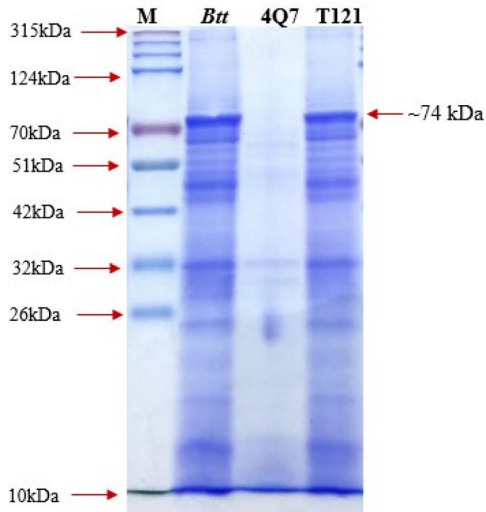

Fig. 2. Protein profile of indigenous *Bacillus thuringiensis* isolate T121 (M, protein marker; Btt, *B. thuringiensis* subsp. *tenebrionis* positive standard; 4Q7, negative standard).

Table 2. Toxicity of spore crystal mixture from *Bacillus thuringiensis* isolate T121 against first-instar larvae of *Henosepilachna vigintioctopunctata*.

Isolate	Regression Equation	Slope ± SE	χ^2	LC_{50} (μg/ml)	Confidence Limits (μg/ml)		LC_{95} (μg/ml)	Confidence Limits (μg/ml)	
					Upper Limit	Lower Limit		Upper limit	Lower limit
T121	$y = 4.6937x + 1.8438$	4.6937 ± 0.651	2.8407	4.675	5.118	4.271	11.620	16.001	8.4390
Btt (standard strain)	$y = 4.8001x + 2.2766$	4.8001 ± 0.582	3.4609	3.636	3.978	3.324	8.753	11.183	6.850

significant toxicity toward Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) larvae.

Specifically, Cry proteins exhibit toxicity against lepidopteran larvae (Cry1, 130–140 kDa), both lepidopteran and dipteran larvae (Cry2, 71 kDa), coleopteran larvae (Cry3, 66–77 kDa), and dipteran larvae (Cry4, 125–145 and 68 kDa), as documented by Höfte and Whiteley (1989). Besides the *cry3* gene found in isolate T121, binary toxins *vip1* and *vip2* also were identified and confirmed through Sanger sequencing with gene-specific primers. However, in SDS-PAGE, a prominent wide band around 74 kDa, encoding the Cry3Aa protein, was observed. No protein associated with the Vip1/Vip2 could be detected. Bi et al. (2014) also reported that Bt strain HBF-18 contains *vip1Ad1* and *vip2Ag1* genes in addition to *cry8Ga*, which were undetected in SDS-PAGE. However, cloning and expression of *vip1Ad1* and *vip2Ag1* revealed binary toxin activity against Scarabaeoidea larvae. The DNA sequences of *cry3*, *vip1*, and *vip2* genes from T121 exhibit a high degree of similarity to the previously documented sequences.

Insecticidal activity against *H. vigintioctopunctata*. The Bt isolate T121 had a median lethal concentration (LC_{50}) of 4.675 μg/ml (95% fiducial limit [95% FL] = 5.118–4.271 μg/ml) against first-instar larvae of *H. vigintioctopunctata*, whereas the reference strain Btt had an LC_{50} of 3.636 μg/ml (95% FL = 3.978–3.324 μg/ml) (Table 2; Fig. 3). According to Park et al. (2009), the LC_{50} for the Colorado potato beetle was 3.56 μg/ml (95% FL = 2.41–4.97 μg/ml) for the Cry3Aa crystal suspension. In bioassays of the Cry7Ab3 protoxin against *H. vigintioctopunctata*, Song et al. (2012) determined an LC_{50} of 0.209 mg/ml for second-instar larvae and 1.711 mg/ml for third-instar larvae. The engineered Bt strain HD7AB exhibited significant toxicity against second-instar *H. vigintioctomaculata* larvae, with an LC_{50}

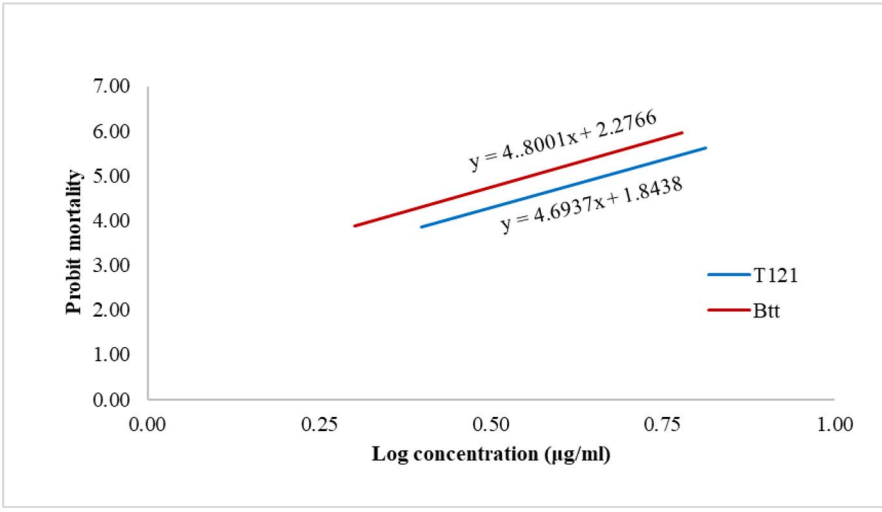

Fig. 3. Probit analysis of the toxicity exhibited by *Bacillus thuringiensis* isolate T121 and *B. thuringiensis* subsp. *tenebrionis* (Btt) against first-instar larvae of *Henosepilachna vigintioctopunctata*.

of 0.779 μg/μl (Wang et al. 2010). Cry3Aa has been documented for its toxicity against various pests, including *L. decemlineata*, *Alphitobius diaperinus* (Panzer), *Diabrotica virgifera virgifera* Leconte, *Acanthoscelides obtectus* (Say), *Cylas puncticollis* Boheman, and *Cylas brunneus* (F.) (Ekobu et al. 2010, Li et al. 2013, Park et al. 2009, Rodríguez-Gonzalez et al. 2020). Our results demonstrate toxicity of Cry3Aa against *H. vigintioctopunctata* first instars, thereby expanding the list of target insect species for this toxin. The characterization of native Bt isolate T121, encompassing morphological, genetic, and functional aspects, underscores its potential as a biopesticide with enhanced insecticidal efficacy against coleopteran pests. Confirming the presence of essential toxin genes, protein expression, and significant mortality rates against *H. vigintioctopunctata* highlights Bt isolate T121 as a promising candidate for integrated pest management strategies.

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