Sujayanand *et al.*
Egyptian Journal of Biological Pest Control (2023) 33:76
https://doi.org/10.1186/s41938-023-00722-y

Egyptian Journal of Biological Pest Control

RESEARCH

Open Access

Characterization of entomotoxic and nematotoxic genes from indigenous *Bacillus thuringiensis* strains and their biocontrol potential

G. K. Sujayanand[1*], Sonika Pandey[1], R. Jagadeeswaran[1], Anup Chandra[1], Vaibhav Kumar[2], Sachin Dubey[1] and Jyotirmay Dubey[1]

Abstract

Background The gram pod borer (*Helicoverpa armigera* Hubner), Bihar hairy caterpillar (*Spilosoma obliqua* Walker), tobacco caterpillar (*Spodoptera litura* Fabricius) and root knot nematode (*Meloidogyne incognita* (Kofoid and White) Chitwood) are the major insect pest and nematodes infesting mungbean, urdbean and other legumes. *Bacillus thuringiensis* (*Bt*) is a promising biocontrol agent that helps in managing the above pest. Deploying *Bt* biopesticides instead of chemical pesticides in legume cultivation enhances the soil health substantially. The present study envisages morphological, molecular cum biochemical characterization, entomotoxicity and nematotoxicity potential of four indigenous *Bt* strains (Ak_2.IIPR, F8.IIPR, F5.IIPR and F6.IIPR) along with HD1 (standard check from BGSC).

Results The SEM micrograph analysis of above four *Bt* strains along with F1.IIPR revealed the presence of different combinations of insecticidal crystal proteins, viz. cuboidal crystal (CC), bipyramidal crystal (BC), spherical crystal (SC) and irregularly pointed crystal (IC). This was further confirmed by PCR amplification of *Cry1*, *Cry2Aa*, *Cry2Ac*, *VIP3A* and *chi36* gene-specific primers and their phylogenetic analysis. F8.IIPR and Ak_2.IIPR recorded the highest toxicity index against second-instar *S. obliqua* larvae (292.56 and 174.55), *H. armigera* (150.00 and 113.65) and *S. litura* (210.71 and 114.19) based on probit analysis. F5.IIPR (91.67%) and F8.IIPR (89.00%) recorded the highest juvenile mortality against *M. incognita*, followed by Ak_2.IIPR (70.33%). The protein profiling of supernatant of terrific broth inoculated with F8.IIPR indicated the presence of VIP3A and Cry1I.

Conclusion The present study concluded that F8.IIPR and Ak_2.IIPR were potential *Bt* strains isolated from Indo-Gangetic plains which is having both entomocidal and nematicidal properties. Further, these strains were deposited at national repository, ICAR-NBAIM with the following accession no.: NAIMCC-SB-065 and NAIMCC-SB-066, respectively. These strains can be formulated as microbial biopesticide.

Keywords Insecticidal genes, Exochitinase gene, Toxicity index, Nematicidal genes, *Bt* DNA gyrase, SEM analysis

Background

Mungbean (*Vigna radiata* (L.) R. Wilczek*var. radiata*) is an important legume crop cultivated in South East Asia predominantly. The global area is 7.3 million hectares (Mha) with an average yield of 721 kg/ha (Nair and Schreinemachers 2020). In India, it is cultivated in 4.52Mha with a production of 2.51 million tons

*Correspondence:
G. K. Sujayanand
sujayanand.gk@icar.gov.in
[1] Division of Crop Protection, ICAR-Indian Institute of Pulses Research, Kanpur 208024, India
[2] Division of Basic Science, ICAR-Indian Institute of Pulses Research, Kanpur 208024, India

Springer Open

© The Author(s) 2023. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

during 2019–2020 and an average yield was 544.25 kg/ha (Anonymous 2022). There is an ample scope to increase mungbean productivity by reducing the losses inflicted by key insect pest and nematodes. It is affected by an array of insect pest and nematodes. Polyphagous lepidopteran insects such as *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae), *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) and *Spilosoma obliqua* Walker (Lepidoptera: Erebidae) were infesting severely in legume crops in India, Philippines, Cambodia, Lao PDR and Indonesia (Sequeros et al. 2021).

Root knot nematodes (RKN), *Meloidogyne* spp. are capable of damaging a broad range of crops and causing serious yield losses. It causes heavy yield loss amounting to 18–90% in mungbean under congenial environmental conditions (Singh et al. 2020). This genus has around 98 species among which *Meloidogyne incognita* (Tylenchida: Heteroderidae) is very common and it causes root galls in almost all vascular plants (Jones et al. 2013). This is very difficult to control due to their quick generation time, high reproduction rate, sedimentary and endophytic nature (Engelbrecht et al. 2018). So, the main issue in the present scenario is to control these biotic factors so that crop yield losses can be prevented. Chemical insecticides and nematicides are generally used for managing this pest and RKN, but due to the negative impact of these pesticides on environmental and non-targets, the need for efficient biocontrol agents is increasing (Masson et al. 1998).

Bacillus thuringiensis Berliner (*Bt*) is an eco-friendly, target-specific microbial biological control agent. It helps in sustainable management of insects and nematodes. In India, only four *Bt kurstaki* strains (HD1 serotype 3a3b, Z-52 3a3b, A-97 serotype H-3a and DOR Bt-1 serotype-3a3b3c) were registered for commercial use with CIBRC (Central Insecticides Board and Registration committee). Out of four *Bt* strains, only DOR *Bt*-1 is indigenous *Bt* strain and most of the available *Bt* products (Delfin, Dipel, Thuricide, Halt, Biobit, Bactospeine, Agree, etc.) were produced from imported *Bt* strains (Ramanujam et al. 2014). Native strains were known for their higher toxicity and greater efficiency in managing the native insect pests because of coexistence and evolution (Hassan et al. 2021). The present experiments were carried out to study the entomotoxic and nematotoxic potential of indigenous *Bt* strains isolated from Indo-Gangetic plains against three polyphagous lepidopteran pests and root knot nematode, *M. incognita* infesting mungbean. Mungbean and other legumes were known for its role in enhancing the soil microbial diversity and thereby improving soil health by biological N fixation, P solubilization, organic carbon enhancement, etc.; when *Bt* biopesticides were deployed in place of synthetic chemical insecticides it will help conserving the microbial diversity in legume-based cropping systems (Ananda et al. 2022). Endospores and CRY proteins are the major active ingredients in any *Bt* biopesticides, and the cry protein alone accounts for more than 20–30% of total cell mass of *Bt* (Agaisse and Lereclus 1995). Endospore formation and cry protein accumulation occur during the lag phase of *Bt* growth. Classification of CRY proteins can be done on the basis of amino acid sequence and host specificity (Asokan et al. 2012). The only exception in cry protein is Cry1I because it is an excretory protein and it is active against both lepidopteran and coleopteran (Tailor et al. 1992). Hence, in the present study, five native *Bt* strains insecticidal genes were subjected to polymerase chain reaction (PCR) screening, scanning electron micrograph (SEM) analysis and bioassay against major pests for documenting its insecticidal genes diversity and its efficacy.

Methods

Morphological and biochemical characterization

The physio-morphological characteristics, viz. colony color, appearance, growth characters and spore morphology, were recorded 7 days after incubation at 30 °C, following standard identification protocols (Logan et al. 2009) for 12 native *Bt* strains maintained in Bio-ecology Lab, ICAR-Indian Institute of Pulses Research (ICAR-IIPR), Kanpur, India. Similarly, biochemical characterizations for 12 *Bt* isolates along with four standard *Bt* strains, viz. *Bt* subsp. *kurstaki* HD1, *Bt* subsp. *aizawi* HD133, *Bt* subsp. *israelensis* ONR60A and *Bt* subsp. *morrisoni* biovar *tenebrionis* (kindly supplied by Dr D. R. Zeigler from BGSC), was done with Hi-Bacillus kit (KB013) from M/s HiMedia Laboratories Pvt. Ltd., Mumbai, India, as per the instructions provided.

Hemolytic assay

Himedia sheep blood agar base with fresh sheep erythrocytes (RBC) was used to carry out this assay as per the instructions provided. After incubation at 30 °C for 24 h, the Petri plates were examined for the presence or absence of hemolytic zone surrounding the bacterial colonies (Ichikawa et al. 2008).

Spore crystal mixture (SCM) extraction and *Bt* endospore enumeration

A loop full of pure culture of four potential *Bt* strains (Ak_2. IIPR, F8.IIPR, F5.IIPR and F6.IIPR and HD1) based on preliminary study (Sujayanand et al. 2021) were inoculated in a 250-ml conical flask containing 100 ml of nutrient broth and incubated in a shaker for 4 days at 30 °C. The spore crystal mixture (SCM) was isolated as described by Zothansanga et al. (2016). The SCM was serially diluted up to 10^{-9} cfu/ml, and its colony-forming units (CFU)

were estimated by spread plate technique by replicating each dilution thrice and the plates were incubated at 30 °C overnight.

Scanning electron micrograph (SEM) analysis of SCM

The spore crystal mixture of *Bt* strains, viz. Ak_2.IIPR, F8.IIPR, F5.IIPR, F6.IIPR and F1.IIPR, were diluted from 10^{-4} to 10^{-6} cfu/ml according to the endospore and crystal concentration in the respective strain. Then, the respective strains were subjected to SEM analysis as described by El-kersh et al. (2012) with slight modifications given in the following. For SEM analysis, a small aliquot of diluted SCM was taken and smeared on a cover glass, followed by vacuum evaporation for 45 min. The cover glass was then placed on carbon stub and subjected to sputter coating with gold (Hitachi, MC 1000) for 5 min. The gold-coated sample was placed in chamber of SEM (Hitachi, S-3400N), and secondary electron/backscattered electron micrographs were recorded by passing 1500kv current. The SEM micrographs were taken at ICAR-National Bureau of Agriculturally Important Microorganism (ICAR-NBAIM), Mau, India.

Insect bioassay

The toxicity potential of four native *Bt* strains (identified through range finding test), viz. Ak_2.IIPR, F8.IIPR, F5.IIPR and F6.IIPR, was compared along with the standard *Bt* strain, i.e., *Bt* subsp. *kurstaki* HD1, by artificial diet contamination method (Li and Bouwer 2012). All the bioassay experiments were laid out in completely randomized design (CRD). Three polyphagous lepidopteran pests of mungbean, viz. Bihar hairy caterpillar (BHC), *S. obliqua*, pod borer (PB), *H. armigera*, and tobacco caterpillar (TC), *S. litura*, were maintained in Bio-ecology Lab, ICAR-Indian Institute of Pulses Research, Kanpur. The second-instar larvae of the above-mentioned three insects were subjected to insect bioassay by artificial diet contamination method with different SCM concentrations (4 for BHC; 5 for PB; and 3 for TC) of *Bt* strains. The insect mortality was recorded up to 7 days after treatment, and percent mortality for each strain was estimated. The lethal concentration of each *Bt* strain SCM (in cfu/ml) against these insects was estimated using probit analysis in SAS 6.2 by using PROC GLM procedure. The potency of the strains was compared as per the following formula (Sun and Jhonson 1960):

Toxicity index (T.I) of *Bt* strain = [LC_{50} of standard strain/LC_{50} of test strain] × 100.

Chitinolytic activity and bioassay against root knot nematodes (RKN)

Colloidal chitin preparation

One hundred grams of chitin flakes (shrimp shell, Sigma) was added slowly to 1.1.8 l concentrated HCl in a glass jar, and it was mixed gently for 3 h on a magnetic stirrer. This solution was then filtered by using eight layers of cheese cloths to 5 l of pre-chilled, distilled water with constant mixing to allow colloidal chitin to precipitate. This was incubated in a refrigerator at 4 °C for overnight. A dense white precipitate was formed and centrifuged at 10,000 rpm for 10 min at 4 °C. The precipitate was then washed in cold, distilled water repeatedly until the pH of the wash reached 5.5. The supernatant was discarded, and the colloidal chitin was then kept in a refrigerator for future use (Hsu and Lockwood 1975).

Chitinolytic activity

Colloidal chitin-enriched broth was prepared by adding 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.5 g yeast extract and 5 g colloidal chitin in 1 l of distilled water. *Bt* strains were inoculated in 50 ml of autoclaved chitin-enriched broth in a 100-ml Erlenmeyer flask and incubated at 26 °C for two days. The suspensions were centrifuged, and the supernatant was filtered through MILLEX GV bacterial filter 0.22 µm (Merck). Colloidal chitin suspensions containing 1% (weight per volume) purified colloidal chitin in 100 mM of acetate buffer at pH 5.0 were used as chitin substrate (Vessey and Pegg 1973). The chitinolytic activity of five *Bt* strains, viz. Ak_2, F8, F5, F4 and F1, was evaluated by using bacterial cultural filtrates containing crude chitinase solution and colloidal chitin suspension as a substrate which was incubated at 37 °C for 12 h, and spectrophotometric OD value of the solution was recorded at 420 nm.

RKN bioassay

One milliliter of cultural filtrates of all the strains with three replications was taken in 12-well tissue culture plates and distilled water as the control. Hundred freshly hatched second-stage juveniles (J2) were inoculated in each treatment. After 48 h, the dead nematodes were counted and kept separately in water to confirm whether juveniles were dead or nematostatic. The percent mortality was calculated based on the following formula:

Percentage J2 mortality = (No. of dead J2 /Total No. of juveniles) × 100.

The single egg mass culture of root knot nematode maintained at Nematode Biocontrol Laboratory, ICAR-IIPR, Kanpur, India, was deployed for challenging the chitinase supernatant obtained from the above-mentioned strains against J2 stage of root knot nematodes (RKN), i.e., *Meloidogyne* sp. The mortality was recorded for up to 7 days after treatment, at a time interval of every 24 h.

DNA isolation, PCR amplification of *gyrB*, *Cry*, *Vip3Aa* and *chi36* genes and its phylogeny

The DNA of 12 *Bt* strains was extracted using Nucleopore®g DNA fungal bacterial mini kit (NP-7006D). The *DNA gyrase* gene (*gyr B*) was amplified for the 12 *Bt* strains as reported by Subbanna et al. (2018), except a slight modification in the annealing temperature which was set up at 50 °C for the current experiment.

The *cry* gene content of six *Bt* strains (Ak_2.IIPR, F8.IIPR, F5.IIPR, F6.IIPR, F4.IIPR and F1.IIPR) was identified by using oligonucleotide primers, viz. Gral-*cry1*, Gral*cry2*, spe-*cry2Aa*, spe-*cry2Ab* and spe-*cry2Ac*, through PCR analysis as reported by Mendoza et al. (2012). The PCR conditions, followed for amplifying, were slightly modified from Vilas-Bos and Lemos (2004) for specific *cry* gene amplification with respect to annealing temperature between 50 and 53.7 °C according to the *Bt* strain requirement. The *vip3* gene was amplified with primers and the PCR conditions as reported by Franco-Rivera et al. (2004) for five *Bt* strains (Ak2.IIPR, F8.IIPR, F5.IIPR, F6.IIPR and F1.IIPR).

The exochitinase (*chi36*) gene was amplified by using the primers reported by Arora et al. (2003). The PCR program for amplifying the exochitinase gene is given as follows: 94 °C for 5 min, 94 °C for 1 min, 50.2 °C for 1 min, 72 °C for 1 min and 30 s for 35 cycles and 72 °C for 10 min. The program will yield an amplicon with a size of 1083 bp. The PCR products were subjected to Sanger dideoxy sequencing by M/s Eurofins (P) ltd and M/s Xceleris (P) ltd. The above gene sequences were trimmed using BLAST analysis with the help of Bioedit and Finch TV 1.4.0 and then submitted to the NCBI database. The phylogenetic analysis of the four genes, viz., *DNA gyrase*, *cry 1*, *cry2* and *vip3A,* was performed using MEGA7 by a neighbor-joining method.

VIP3 protein profiling

A 12-h-old nutrient broth culture of F8.IIPR was used as mother inoculum for vegetative insecticidal protein (VIP3) production in 100 ml of Terrific broth incubated for 19 h. The VIP3 protein present in the supernatant was precipitated, purified and dialyzed as described by Sattar et al. (2008). The concentrated protein was stored at − 20 °C until it was subjected to SDS–PAGE analysis.

Results

Morphological attributes and biochemical characteristics of 12 *Bt* strains

The growth of 12 *Bt* strains in nutrient agar (NA) was recorded 7 days after incubation at 25 °C in a BOD incubator. The highest width recorded was 1.06 cm (RoT61.IIPR), followed by 0.96 cm (Pa2.IIPR) and 0.9 cm (J3.IIPR, Ak_2.IIPR) (Table 1). The lowest width (0.56 cm) was recorded from SgH5.IIPR, F4.IIPR and F6.IIPR. Nine strains showed irregular, continuous growth, whereas two strains recorded discontinuous growth (F4.IIPR and RoT61.IIPR) and one strain recorded regenerative growth. All the strains had exhibited off-white color. Almost majority of *Bt* strains were opaque with moist appearance, except Ak2.IIPR and F4.IIPR, wherein they have dry opaque appearance. Four *Bt* strains had shown lobate margin, while four *Bt* strains had undulated margins. All 12 *Bt* strains were gram positive, were sporulating and formed crystal protein (Table 1). The biochemical reactions performed with Hi-Bacillus kit showed that all the 16 (12 native *Bt* + 4 standard *Bt*) were catalase positive (Table 2), while for malonate test, all the *Bt* strains were negative, except J3.IIPR and F4.IIPR, and for Voges–Proskauer's test, all strains were negative. Regarding utilization of citrate as the sole carbon source, all the isolated strains were negative, except J3.IIPR, F4.IIPR and F8.IIPR. For ONPG test, only F8 was positive and the remaining strains showed a negative reaction. Nitrate reduction test was found to be positive for all the strains, except F8.IIPR and J3.IIPR. Three *Bt* strains, viz. Ak_2.IIPR, SgH_5.IIPR and F6.IIPR, were negative for arginine production. For sugar reduction test, all the strains were negative for mannitol and arabinose, except F8.IIPR, which showed a positive reaction, whereas for glucose all the strains were positive, except F1.IIPR, F4.IIPR and HD1.

Hemolytic assay

No hemolytic zone was recorded from 12 *Bt* strains in sheep blood agar. Hence, these are concluded as non-hemolytic *Bt* strains.

SCM extraction and endospore enumeration

The spore crystal mixtures of four *Bt* strains (Ak_2, F8, F5 and F6) and standard strain (HD1) were extracted and stored at − 20 °C for assessing their insecticidal potential and characterizing their crystal protein shape and endospore morphometrics. The endospore enumeration of each strain in different dilutions showed variation among the strains. All the tested four *Bt* strains recorded a higher number of endospores (at a dilution of 10^{-7} and 10^{-9} cfu/ml) than the standard strain. The highest number of endospores (1650 cfu/ml) at 10^{-9} was recorded from F8.IIPR, followed by Ak_2.IIPR, F6.IIPR and F5.IIPR, whereas the lowest endospore count (495 cfu/ml) was recorded from standard strain, HD1 (Fig. 1).

SEM analysis of endospore and crystal protein

The electron micrographs of the five *Bt* strains revealed the presence of different crystal morphologies in single strain such as presence of cuboidal crystal (CC),

Table 1 Morphological characteristics and phenotype of *Bacillus thuringiensis* isolates

S. no.	Strain names	Growth characters	Color	Appearance of the colony	Colony growth after 7 days on NA (cm)	Endospore size (µm)	
						Length	width
1	AK2.IIPR	Regenerative growth	Off white	Dry, opaque, flat colonies	0.9 ± 0.15	1.8	1.3
2	F4.IIPR	Discontinuous growth	Cream yellow	Dry, opaque colonies with lobate margins	0.56 ± 0.07	1.9	1.3
3	F5.IIPR	Irregular, continuous growth	Off white	Moist, flat and opaque colonies with lobate margins	0.66 ± 0.09	1.8	2.4
4	SgH5.IIPR	Irregular, continuous growth	Off white	Undulate colony margins, transparent colonies, elevation raised	0.56 ± 0.03	1.8	1.6
5	F1.IIPR	Irregular, continuous growth	Off white	Moist, flat and opaque colonies with flat margins	0.7 ± 0.12	1.8	0.8
6	F6.IIPR	Irregular continuous growth	Off white	Moist, opaque, lobated margined colonies with flat elevation	0.56 ± 0.03	1.9	1
7	F8.IIPR	Irregular, continuous growth	Off white	Moist, opaque, flat colonies with lobate margins	0.8 ± 0.06	1.75	1.2
8	J3.IIPR	Irregular continuous growth	Off white	Opaque colonies. Colony surface smooth. Margin undulates. Elevation flat	0.9 ± 0.07	1.55	0.9
9	B1.IIPR	Irregular, continuous growth	Off white	Viscous, opaque, elevation raised, margin undulates. Colony surface smooth	0.66 ± 0.15	2.2	1.9
10	RoT61.IIPR	Discontinuous, circular growth	Off white	Moist, opaque colonies. Colony surface continuous	1.06 ± 0.09	1.8	1.5
11	Pa.IIPR	Irregular, continuous growth	Off white	Elevation raised, margin undulates, moist, opaque colonies	0.76 ± 0.06	1.9	1.3
12	Pa2.IIPR	Irregular, continuous growth	Off white	Moist, opaque colonies. Colony surface smooth. Margin undulates. Elevation flat	0.96 ± 0.13	2.2	1.4
					CD = 0.28		

bipyramidal crystal (BC), spherical crystal (SC) and irregularly pointed crystal (IC) in the case of F8.IIPR (Fig. 2b). Whereas Ak_2.IIPR (Fig. 2a), F6.IIPR (Fig. 2d) and F1.IIPR (Fig. 2e) had three crystal morphologies, viz. BC, CC and SC, F5.IIPR (Fig. 2c) had only two crystal morphologies, viz. CC and BC. The endospore length varied from 1.63 to 2.36 µm; similarly, breadth varied from 0.79 to 1.18 µm. There existed a significant variation among the *Bt* strains with respect to endospore length and breadth (Fig. 2f). Ak_2.IIPR had the largest endospore, while F6.IIPR had the smallest endospore with respect to breadth of endospore.

Insect bioassay

The second-instar Bihar hairy caterpillar, *S. obliqua* was challenged with SCM of four native *Bt* strains and *Btk* HD1. The lowest LC_{50} was recorded from F8.IIPR (1.99×10^3 cfu/ml), followed by Ak_2.IIPR (3.34×10^3 cfu/ml) and F5.IIPR (5.18×10^3 cfu/ml) (Table 3). The HD1 recorded a LC_{50} of 5.18×10^3 cfu/ml against *S. obliqua*. The toxicity index was the highest (292.96) for F8.IIPR, followed by Ak_2.IIPR (174.55) and F5.IIPR (112.55). Among the four *Bt* strains screened against the second-instar *H. armigera* larvae, the lowest LC_{50} was recorded from F8.IIPR (2.22×10^3 cfu/ml), followed by Ak_2.IIPR (2.93×10^3 cfu/ml), while two strains, viz. F6.IIPR (5.06×10^3 cfu/ml) and F5.IIPR (6.82×10^3 cfu/ml), had recorded LC_{50} value higher than the standard check, *Btk* HD1 (3.33×10^3 cfu/ml). This showed that two *Bt* strains were superior to the *Btk* HD1 in killing *H. armigera*. Further, the toxicity index also indicated that F8.IIPR is highly toxic (150.00), followed by Ak_2.IIPR (113.65), F6.IIPR (65.81) and F5.IIPR (48.83).

Based on the preliminary screening of *Bt* SCM against *S. litura*, only two *Bt* strains, viz. F8.IIPR and Ak_2.IIPR, had promising results (data not shown). Among the two *Bt* strains screened against the second-instar *S. litura*, F8.IIPR had recorded the lowest LC_{50} ($2.52X10^3$cfu/ml), followed by Ak_2.IIPR ($4.65X10^3$cfu/ml). The standard check (HD1) had recorded a LC_{50} of 5.31×10^3 cfu/ml. F8.IIPR recorded the highest toxicity index (210.71) against *S. litura,* followed by Ak_2.IIPR (114.19).

Table 2 Biochemical characterization through Hi-Bacillus kit

Sl. no.	Strain Names	Malonate	Vp	citrate	ONPG	Nitrate reduction	Catalase	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalose	Hemolytic agar
1	J3.IIPR	+	−	+	−	−	+	+	−	−	+	−	W +	−
2	F6.IIPR	−	−	−	−	+	+	−	−	−	+	−	+	−
3	Pa2.IIPR	−	−	−	−	+	+	+	+	−	+	−	+	−
4	B1.IIPR	−	−	−	−	+	+	+	+	−	+	−	+	−
5	RoT61.IIPR	−	−	−	−	+	+	+	−	−	+	−	+	−
6	F1.IIPR	−	−	−	−	+	+	+	−	−	−	−	+	−
7	Pa.IIPR	−	−	−	−	+	+	+	+	−	+	−	+	−
8	SgH5.IIPR	−	−	−	−	+	+	−	+	−	+	−	+	−
9	F4.IIPR	+	−	+	−	+	+	+	−	−	−	−	−	−
10	F5.IIPR	−	−	−	−	+	+	+	−	−	+	−	+	−
11	Ak2.IIPR	−	−	−	−	+	+	-	−	−	+	−	+	−
12	F8.IIPR	−	−	+	+	−	+	+	+	+	+	+	+	−
13	4D1 *Bt kurstaki* HD1	−	−	−	−	+	+	+	−	−	−	−	+	−
14	4QI *Bt israelensis* ONR60A	−	−	−	−	+	+	+	+	−	+	−	+	−
15	4AAI *Bt morrisoni biovar tenebrionis*	−	−	−	−	+	+	+	−	−	+	−	+	−
16	4J3 *Bt aizawi* HD133	−	−	−	−	+	+	+	+	−	−	−	+	−

Vp Voges–Proskauer's test, *ONPG* O-nitrophenyl-β-D-galactopyranoside

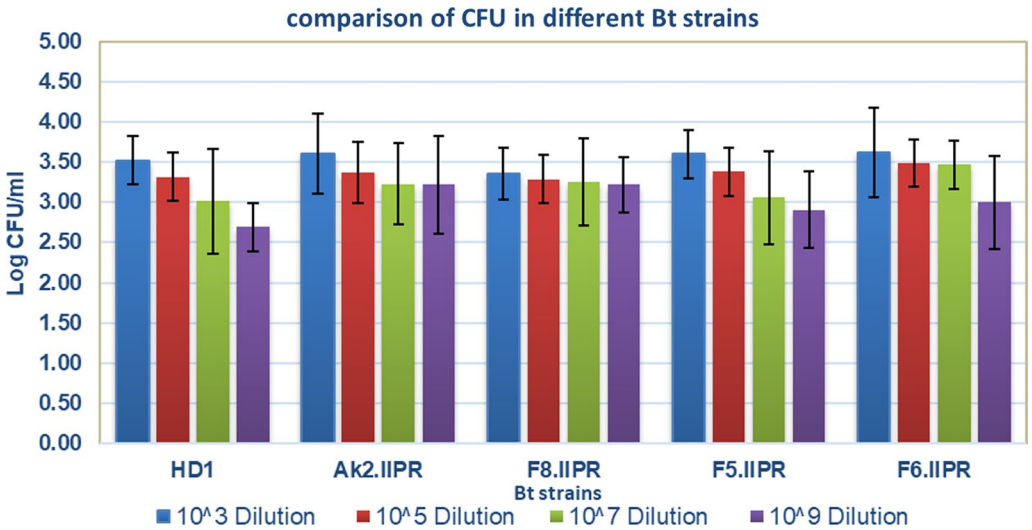

Fig. 1 Comparison of log CFU/ml in different *Bacillus thuringiensis* strains at different dilutions

Chitinolytic activity and bioassay against root knot nematodes

The five *Bt* strains ability to hydrolyze the colloidal chitin by producing a clear zone was recorded on colloidal chitin agar media (Sujayanand et al. 2019). Chitinase activity of *Bt* strains was measured in the presence of 0.2% colloidal chitin and measured by the Schales method. Chitinase activity was measured in U/mg. The maximum chitinase production was observed for F8.IIPR, followed by Ak_2.IIPR. In chitinolytic assay, and the highest activity was recorded from F8.IIPR (0.525), followed by F4.IIPR (0.522), F5.IIPR (0.488), F1.IIPR and Ak_2.IIPR. The percent mortality of root knot nematode juveniles revealed that F5.IIPR recorded the highest mortality, followed by F8.IIPR and Ak_2.IIPR (Table 4).

DNA gyrase, Cry, Vip3Aa and *chi36 genes* phylogeny

The genomic DNA of 12 *Bt* strains was extracted and stored at − 20 °C. The DNA gyrase gene amplification yielded an amplicon of 342 bp for the 12 *Bt* strains. Further phylogenetic analysis indicated that F8.IIPR, Ak_2.IIPR, F1.IIPR, F5.IIPR and F4.IIPR were more close to gyrase gene of *Bt kurstaki* BGSC 4D20 strain (Fig. 3). These strains produced bipyramidal protein. Whereas three *Bt* strains (B1.IIPR, RoT61.IIPR and SgH5.IIPR) showed similarity to *Bt canadensis*, two showed similarity to *Bt pondicheriensis*, one *Bt* strain (F6.IIPR) was more similar to *B. tyoso* strain, while J3.IIPR showed similarity to *Bt entomocidus*.

The *cry1* subtype for the six *Bt* strains [F8.IIPR (MK855068), Ak_2.IIPR (MK843970), F6.IIPR (MK843971), F1.IIPR (MK855069), F5.IIPR (OM141596) and F4.IIPR (OM141595)] was screened by sequencing an amplicon size of 990 bp further phylogenetic analysis and also concluded as *cry1A* (Fig. 4a). Of the six different *Bt* strains screened for *cry2* subtype having 1249 bp from *gral cry2* primers, the sequence results yielded mixed reaction. Hence, we had gone for the gene-specific primers, viz., *cry2Aa* (460 bp), *cry2Ab* (771 bp) and *Cry2Ac* (841 bp). Five *Bt* strains, viz. F8.IIPR (MN548386 and MN548387), Ak_2.IIPR (MT210572 and MT210570), F6.IIPR (MT210573 and MT210571), F1.IIPR (MT210575 and MT210576) and F5.IIPR (MT210574 and OM141597), had both *cry2Aa* (460 bp) and *cry2Ac* (841 bp), while F4.IIPR had only *cry2Ac* (MT210577) (Fig. 4b).

The *Vip 3A* gene was found to be present in all five *Bt* strains, viz. Ak2.IIPR (MF143589), F8.IIPR (MF143591), F6.IIPR (MF143590), F1.IIPR (MK124722) and F5.IIPR (MK214423), based on phylogenetic analysis (Fig. 4c).

The partial exochitinase gene *chi36* was amplified in F8.IIPR (MN991173), Ak_2.IIPR (MN985815), F1.IIPR (MN879415) and F5.IIPR (MN985816) to give a PCR product of 1083 bp when annealed at 50.2 °C.

VIP3 protein profiling

The VIP3 protein from terrific broth was extracted, purified and dialyzed. The SDS–PAGE analysis of the supernatant from F8.IIPR strain reveals the presence of protein with 88 kDa (Fig. 5).

Discussion

The genus *Bacillus* can be divided into five groups based on the phylogenetic analysis of 16S rRNA, viz. *Bacillus cereus*, *B. megaterium*, *B. subtilis*, *B. circulans* and *B. brevis*. The *Bt* falls in *B. cereus* group. The *B. cereus* group has typical colony morphology such

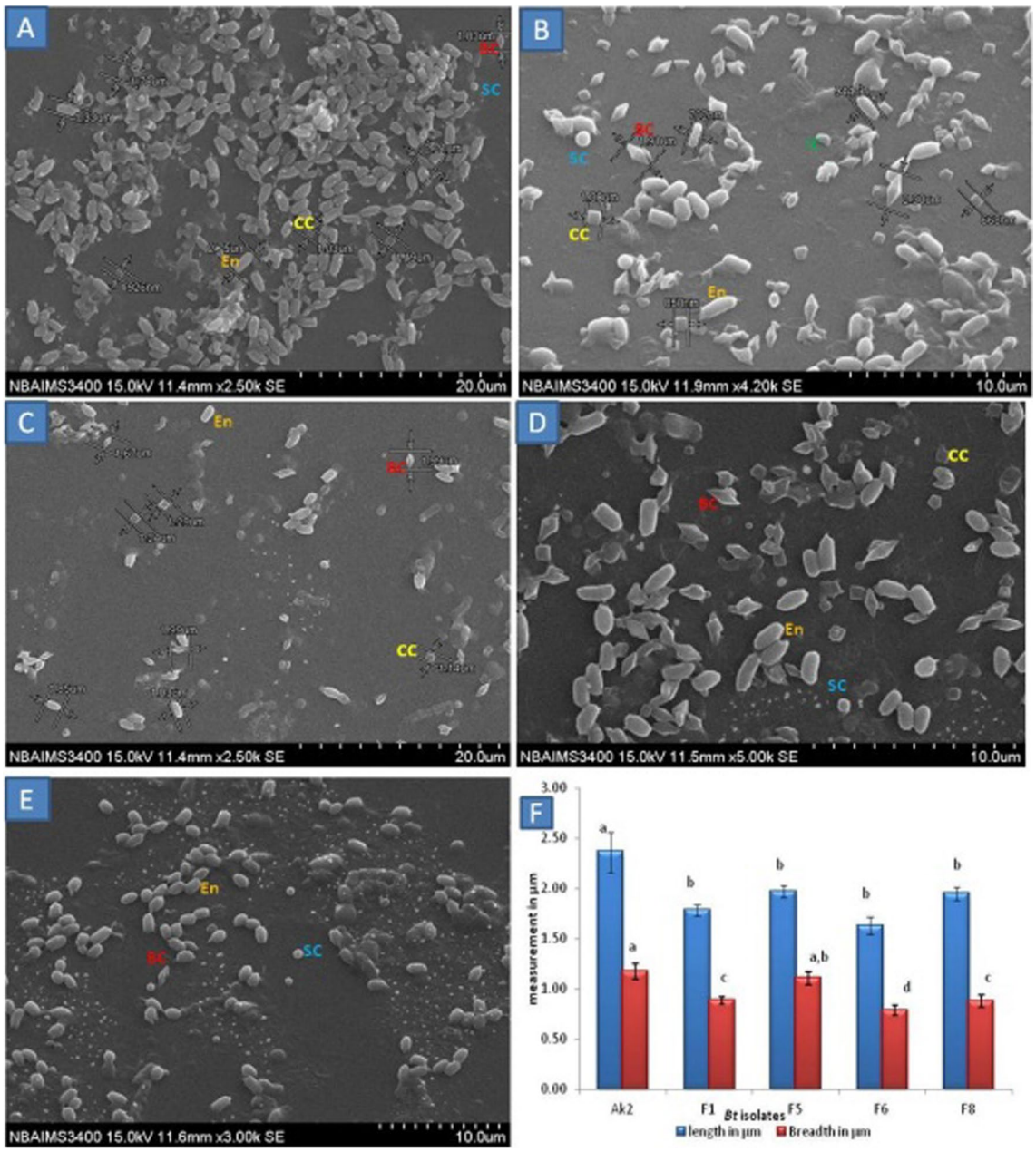

Fig. 2 Scanning electron micrograph (SEM) morphometric analysis of CRY protein. **A** Ak2.IIPR, **B** F8.IIPR, **C** F5.IIPR, **D** F6.IIPR, **E** F1.IIPR and **F** endospore morphometrics; *En* endospore, *CC* cuboidal crystal protein, *BP* bipyramidal protein, *SC* spherical crystal protein, *IC* irregular crystal protein

as off-white color of colonies, rough waxy growth and irregular spreading margins on nutrient agar as reported by Venkatramanamma et al. (2022). All 12 indigenous *Bt* strains exhibited the above-said colony morphological characteristics. The present findings were in congruence with those of Hassan et al. (2021), wherein they had reported majority of *Bt* isolates were off white with wavy margins, dry or moist appearance and flat colony morphology. Apart from recording the colony morphology, the variation in speed of growth in nutrient agar for the 12 *Bt* strains was also recorded by incubating at 25 °C for 7 days. The colony width ranged from 0.56 to 1.06 cm. The present results contradict the findings of Bautista and Teves (2013) who reported a colony growth of < 1 to 4 mm. The reason for lesser growth was that they had incubated for shorter period (24 h) only at 30 °C but the incubation time was high (7 Days) in the present study. Hi-Bacillus™ Identifications

Table 3 Toxicity of *Bacillus thuringiensis* isolates of spore crystal mixture (SCM) against three polyphagous mungbean pests

S. no.	Isolates	LC_{50} (CFUs/ml)	Slope	Pearson χ^2	Degrees of freedom	95% Fiducial limits		LC_{95}(CFUs/ml)	Toxicity index
						Lower	Higher		
Bihar hairy caterpillar, Spilosoma obliqua									
1	HD1	5.83×10^3	0.7807 ± 0.2790	0.53	2	2.92×10^3	1.67×10^5	7.46×10^5	
2	Ak2.IIPR	3.34×10^3	2.0342 ± 0.5090	3.72	2	2.72×10^3	5.21×10^3	2.15×10^4	174.55
3	F8.IIPR	1.99×10^3	6.0384 ± 1.6063	1.84	2	1.87×10^3	2.19×10^3	3.73×10^5	292.96
4	F5.IIPR	5.18×10^3	0.7252 ± 0.3057	0.12	2	2.75×10^3	9.78×10^5	9.61×10^5	112.55
5	F6.IIPR	10.23×10^3	0.8689 ± 0.3839	1.12	2	4.98×10^3	7.85×10^7	7.99×10^5	56.99
Helicoverpa armigera									
1	HD1	3.33×10^3	1.0180 ± 0.2236	1.00	3	2.35×10^3	6.17×10^3	1.75×10^5	
2	Ak2.IIPR	2.93×10^3	2.644 ± 0.3656	3.21	3	2.57×10^3	3.39×10^3	1.23×10^4	113.65
3	F8.IIPR	2.22×10^3	5.904 ± 0.8455	0.72	3	2.09×10^3	2.38×10^3	4.21×10^3	150.00
4	F5.IIPR	6.82×10^3	1.992 ± 0.3420	3.83	3	5.16×10^3	11.19×10^3	4.56×10^3	48.83
5	F6.IIPR	5.06×10^3	2.135 ± 0.6510	7.89	3	3.26×10^3	1.60×10^{10}	29.82×10^3	65.81
Spodoptera litura									
1	HD1	5.31×10^3	4.165 ± 1.6943	0.2034	1	4.70×10^3	7.80×10^3	13.17×10^3	
2	Ak2.IIPR	4.65×10^3	6.312 ± 1.8306	0.0374	1	3.90×10^3	5.04×10^3	8.47×10^3	114.19
3	F8.IIPR	2.52×10^3	3.247 ± 1.37	1.0009	1	718.82	2.96×10^3	8.07×10^3	210.71

Table 4 Chitinolytic activity of *Bacillus thuringiensis strains* and its efficacy against root knot nematode (RKN) juveniles

Bt strain names	OD value @ 420 nm (pooled mean)	Protein concentrations (µg/ml)	Mean % mortality of juvenile RKN*
Ak2.IIPR	0.462	28	70.33 (57.14)
F1.IIPR	0.480	30	56.00 (48.58)
F4.IIPR	0.522	32	55.33 (48.04)
F5.IIPR	0.499	30.5	91.67 (73.31)
F8.IIPR	0.525	32.5	89.00 (70.78)
C.D	0.033	–	10.042
SE(m)	0.01	–	3.146
SE(d)	0.015	–	4.45
C.V	3.574	–	9.148

* Values within parenthesis are arcsine transformed

kit (KB013; HiMedia, India) was deployed by Rajasekhar et al. (2017) for biochemical characterization of *Bt* strains from India and Sudan. The present finding on Voges–Proskauer's and ONPG test was in congruence with the above report. Thus, all these biochemical tests showed existence of variation among *Bt* strains as reported by Martin et al. (2010)

Generally, the dipteran toxic strains, *i.e.*, *Bt israelensis,* have more probability of possessing hemolytic type. Further, they predominantly have β-exotoxin which is responsible for hemolytic activity (Obeidat et al. 2012). The present result is in accordance with those of El-kersh et al. (2012). They also reported the occurrence of non-hemolytic strains (8%) from Saudi Arabia. Further, they had reported only 31% strains were following *Bt kurstaki* biochemical type, which may be the reason for low occurrence of non-hemolytic strain.

The *Bt kurstaki* strain, BA 83B from IMTECH, Chandigarh, produced bigger endospores (length 2.1 ± 0.16 µm and diameter 1.63 ± 0.16 µm) in soybean-supplemented medium than in control (length 1.1 ± 0.13 µm and diameter 0.63 ± 0.07 µm). Thus, the endospore size varies according to the nutrition available (Jisha and Benjamin 2014). Moreover, Elsayed et al. (2014) reported that nutrient requirement varied according to the type of *Bt* strains. The present results are also in accordance with the findings of Elsayed et al. (2014) as there is a variation in endospores (1.63 to 2.36 µm length). Further, the variation in endospore production was also due to genetic variations in the *Bt* strains growth potential as reported by Argolo Filho et al. (2011) who had divided the *Bt* strains into three groups based on their growth rate as fast growers (attaining log phase ~1 h after inoculation), medium growers (attaining log phase ~7 h) and slow growers (attaining log phase 10–11 h) which was measured through spectrophotometrically based OD at 590 nm.

The present result supports the findings of Hassan et al. (2021) wherein they had visualized four different parasporal protein shapes in their *Bt* strains. Further, variation in the crystal protein combination and endospore morphometrics indicates the occurrence of diversity

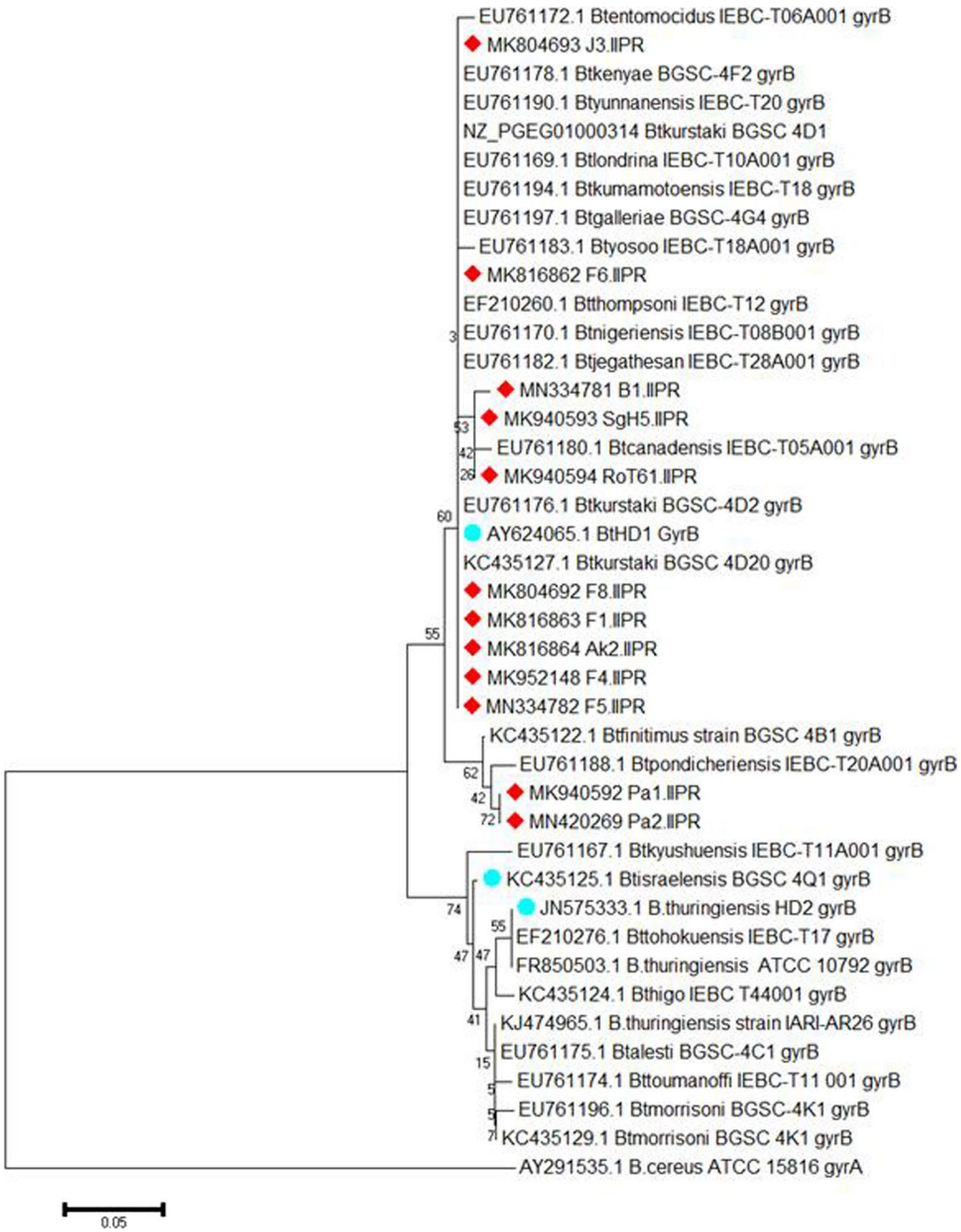

Fig. 3 Phylogenetic analysis for *DNA gyrase* gene of 12 *Bacillus thuringiensis* strains. Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbor-joining method [1]. The optimal tree with the sum of branch length = 0.68590967 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 41 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 119 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4]

among the native *Bt* strains isolated from Indo-Gangetic plains.

The present findings were in congruence with the reports of Roy et al. (2021) wherein they had reported a LC_{50} value of 2.49 X 10^7 cfu/ml against *Culex tritaeniorhynchus* Giles (Diptera: Culicidae). The present findings, i.e., two *Bt* strains (F8.IIPR, Ak_2.IIPR) were having higher toxicity index than HD1 against *H. armigera*, were

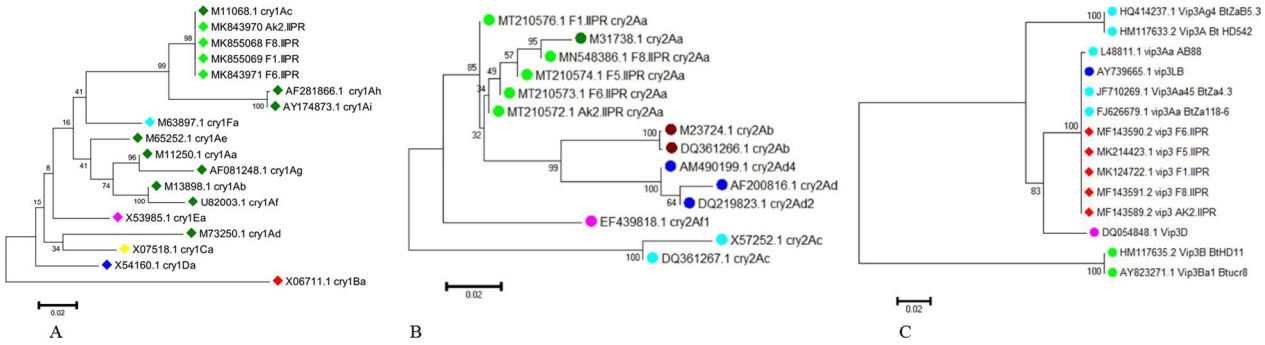

Fig. 4 Phylogenetic analysis for *cry1*, *cry2* and *vip3* genes of potential *Bacillus thuringiensis* strains. **A** Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbor-joining method [1]. The optimal tree with the sum of branch length = 0.52363330 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method [3] and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 665 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4]. 1. Saitou N. and Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425. 2. Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791. 3. Tamura K. and Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512–526. 4. Kumar S., Stecher G. and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870–1874. Disclaimer: Although utmost care has been taken to ensure the correctness of the caption, the caption text is provided "as is" without any warranty of any kind. Authors advise the user to carefully check the caption prior to its use for any purpose and report any errors or problems to the authors immediately (www.megasoftware.net). In no event shall the authors and their employers be liable for any damages, including but not limited to special, consequential or other damages. Authors specifically disclaim all other warranties expressed or implied, including but not limited to the determination of suitability of this caption text for a specific purpose, use or application. **B** Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbor-joining method [1]. The optimal tree with the sum of branch length = 0.34599582 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 4582 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4]. 1. Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425. 2. Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791. 3. Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111–120. 4. Kumar S., Stecher G. and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870–1874. Disclaimer: Although utmost care has been taken to ensure the correctness of the caption, the caption text is provided "as is" without any warranty of any kind. Authors advise the user to carefully check the caption prior to its use for any purpose and report any errors or problems to the authors immediately (www.megasoftware.net). In no event shall the authors and their employers be liable for any damages, including but not limited to special, consequential or other damages. Authors specifically disclaim all other warranties expressed or implied, including but not limited to the determination of suitability of this caption text for a specific purpose, use or application. **C** Evolutionary relationships of taxa. The evolutionary history was inferred using the neighbor-joining method [1]. The optimal tree with the sum of branch length = 0.35582526 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura two-parameter method [3] and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 858 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4]. 1. Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425. 2. Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791. 3. Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111–120. 4. Kumar S., Stecher G. and Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870–1874. Disclaimer: Although utmost care has been taken to ensure the correctness of the caption, the caption text is provided "as is" without any warranty of any kind. Authors advise the user to carefully check the caption prior to its use for any purpose and report any errors or problems to the authors immediately (www.megasoftware.net). In no event shall the authors and their employers be liable for any damages, including but not limited to special, consequential or other damages. Authors specifically disclaim all other warranties expressed or implied, including but not limited to the determination of suitability of this caption text for a specific purpose, use or application

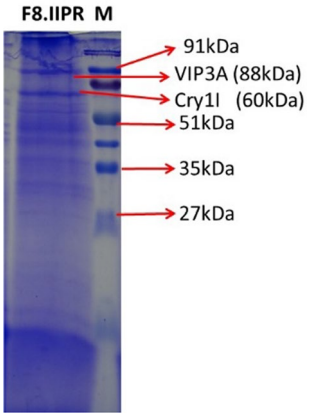

Fig. 5 Polyacrylamide gel electrophoresis (PAGE) profiling of vegetative insecticidal protein (VIP3A) in F8.IIPR strain

supported by the reports of Lone et al. (2017) wherein they had reported a native *Bt* strain (JK12) from Himalayas with a higher toxicity index than HD1 against *H. armigera*. Similarly, in Sri Lanka, Baragamaarachchi et al. (2019) reported that native *Bt* strain (AB1) was highly toxic to Dipel® resistant Diamond back moth, *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae). Thus, native strains were more toxic to locally occurring insect pest.

The present result corroborates with findings of Lee et al. (2015) wherein they also found LC_{50} of *Bt* strains varied from 1.9 X 10^3 to 9.8 X 10^5 cfu/ml. The present results contradict findings of Murali Krishna et al. (2018) wherein they had reported that HD1 (9.59 X 10^4 cfu/ml) is the best strain when compared with LC_{50} of 21 native *Bt* isolates (9.76 X 10^4 to 5.67 X 10^6 cfu/ml) against third-instar *S. litura*. The present result supports the findings of Mohan et al. (2014) wherein they had reported *S. litura* as highly tolerant among the three noctuids (*H. armigera*, *S. litura* and *Sesamia inferens* Walker (Lepidoptera: Noctuidae)) screened against 10 *Bt* strains. In the present study also, *S. litura* has exhibited the highest tolerance [highest LC_{50} (F8.IIPR: 2.52 X 10^3 cfu/ml) than *H. armigera* (2.22 X 10^3 cfu/ml) and *S. obliqua* (1.99 X 10^3 cfu/ml)] among three polyphagous insects studied (Table 3). The probable reason for high tolerance of *S. litura* might be over-digestion of cry protoxin by midgut protease, which was shown in *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) by Ogiwara et al. (1992). Similarly, Hernandez (1988) also had reported that among 13 *Bt* serovars tested against neonates of *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae), only four serovars, viz. *Bt kenyae*, *Bt tolworthi*, *Bt aizawai* and *Bt kurstaki*, had recorded 80 to 100% mortality by diet contamination method. The variation in toxicity level among the strains for three different polyphagous lepidopteran pest stated above is due to functional and toxicological diversity of *cry* genes they harbor and differences in the copy numbers of *cry* genes present, expression pattern and expression levels (Sujayanand and Pandey 2020). Further, the difference in the receptors present in these three insects' midgut also contributes to the variation in their toxicity. Another important factor that contributed to variation in toxicity among the strains was their spore and toxin concentration. Mohan et al. (2014) had already confirmed that *Bt kurstaki* endospores act synergistically with the Cry 2A against *Bt*-resistant population of *Plutella xylostella*.

Researchers all over the world are trying to find biological control measures for *M. incognita* management as chemical nematicides are harming the agro-ecosystem by their toxic residues. There are many bacteria with nematicidal activity such as *Pseudomonas fluorescens* (Flugge 1886) Migula, *P. aeruginosa* Migula, *Bacillus thuringiensis* and *B. firmus* Bredemann and Werner. *P. fluorescens* kills soybean cyst nematode by producing 2,4-diacetylphloroglucinolc (Siddiqui and Shaukat 2003). *Corynebacterium paurometabolu* inhibited nematode egg hatching by producing hydrogen sulfide (Meena and Pimentel 2002). In the present study, F8.IIPR and F5.IIPR exhibited the highest nematicidal activity. Mohammad et al. (2008) studied the biocontrol efficacy of *Bt* against *M. incognita* and observed a reduction in egg masses and egg number as compared to the control. *Bt* DB27 was found to produce two novel proteins, namely Cry21Fa1 and Cry21Hai, which were found to be effective against *Caenorhabditis elegans* Maupas (Iatsenko et al. 2014).

The present result supports the findings of Lee et al. (2015) wherein bipyramidal protein-producing isolates CAB565 and 566 were similar to *Bt kurstaki* KB098 strain and *Bt kurstaki* KB099 strain.

DNA gyrase is more reliable in finding the phylogeny of native *Bt* isolates based on their ecological and evolutionary relationships between *Bt* isolates (Shikov et al. 2021). The phylogenetic analysis of DNA gyrase confirmed that F8.IIPR, Ak_2.IIPR, F1.IIPR, F5.IIPR and F4.IIPR were more close to gyrase gene of *Bt kurstaki* BGSC 4D20 strain and they form a single clade, while F6.IIPR and J3.IIPR were showing phylogeny with different *Bt* serovars. The present report confirms the report that *cry1A* and *cry2A* genes occurred in high frequency (90.61%) by various workers. The *vip3A* gene occurred in medium frequency (40.70%) native *Bt* strains of Thailand (Boonmee et al. 2019). The difference in the toxicities of *Bt* strains may be related to the difference in the composition of crystals protein. Chak et al. (1994) reported a new *Bt* strain with cry l (Ab), cry IC and cry ID genes, and its effectiveness was due to variation in genetic composition of *cry* genes. The combined chitinolytic activity and

entomocidal activity of potential *Bt* strains in the present study imply their utility in deploying them in the integrated pest management as an effective biocontrol agent.

The molecular weight of protein bands that occur in the supernatant corresponds to that of VIP3A as reported by Argolo Filho et al. (2011) wherein they had reported that *Bt* supernatant possesses various secreted entomotoxins with an expected size of ~88 kDa and ~60 kDa corresponding to Vip3A and Cry 1I. Interestingly, the VIP3 protein profiling using supernatant from F8.IIPR also had confirmed the presence of Cry1I as reported by Argolo Filho et al. (2011). Thus, the present work identifies the potential native *Bt* strains (F8.IIPR & Ak_2.IIPR) for managing three polyphagous lepidopteran pests and root knot nematode along with morphological, biochemical and molecular characterizations of pesticidal genes such as *cry1*, *cry2Aa*, *cry2Ac*, *vip3A* and *chi36*.

Conclusion

The present study concluded that two indigenous *Bt* strains, namely F8.IIPR and Ak_2.IIPR, as a potent microbial biocontrol agent among the four indigenous *Bt* strains, were screened against three polyphagous insect pest, viz., *S. obliqua*, *H. armigera* and *S. litura*. The PCR screening and phylogenetic analysis of entomotoxic and nematotoxic genes (*Cry1*, *Cry2Aa*, *Cry2Ac*, *VIP3A* and *chi36*) revealed that the two potential *Bt* strains had multiple insecticidal cum nematicidal genes. Further, this was confirmed by the presence of bipyramidal, cuboidal and spherical crystal protein in SEM micrographs. Further, exochitinases from these strains were found to exhibit toxicity against polyphagous nematode, *M. incognita* also. The SDS–PAGE profiling of F8.IIPR supernatant reveals the presence of CRY1I, which is a special CRY that is secretory in nature along with VIP3. The two potential *Bt* strains F8.IIPR and Ak_2.IIPR were submitted in the National Agriculturally Important Microorganism Culture Collection [NAIMCC], ICAR-NBAIM, with the accession numbers NAIMCC-SB-065 and NAIMCC-SB-066, respectively. Further, the *Bt* strains F1.IIPR, F4.IIPR, F5.IIPR and F6.IIPR were submitted with the following accessions, NAIMCC-B-03049, NAIMCC-B-03050, NAIMCC-B-03051 and NAIMCC-B-03052, respectively. These strains have the potential for further downstream processing to develop as biopesticide for polyphagous lepidopteran pest of this region which will help in conserving the agro-ecological diversity and reduces the dependence of harmful insecticides. Studying the metabolomics of these *Bt* strains will help us in better understanding about CRY1I and VIP3 toxins synergistic activity against polyphagous lepidopteran insects, and it can be extrapolated for genetic engineering of third-generation insect transgenics (*i.e.*, plants with cry1I/VIP3 toxins). This will also assist in development of liquid biopesticides containing both CRY and VIP3 toxins for management of lepidopteran insects. Further investigation is needed on differences in the gut proteases of the three polyphagous lepidopteran insects, which will help in better understanding of evolution of *Bt* resistance as these three insects had varying degrees of *Bt* susceptibility.

Abbreviations

Bt	*Bacillus thuringiensis*
ICAR	Indian Council of Agricultural Research
NAIMCC	National Agriculturally Important Microorganism Culture Collection
NBAIM	National Bureau of Agriculturally Important Microorganism
IIPR	Indian Institute of Pulses Research
SAS	Statistical Analysis Software
BGSC	Bacillus Genetic Stock Centre
SEM	Scanning electron micrograph
kv	Kilovolt
ml	Milliliter
LC_{50}	Lethal concentration 50%
OD	Optical density
nm	Nanometer
HCl	Hydrochloric acid
rpm	Revolutions per minute
min	Minutes
mM	Millimolar
g	Grams
SCM	Spore crystal mixture
CFU	Colony-forming units
BHC	Bihar hairy caterpillar
TC	Tobacco caterpillar
PB	Pod borer

Acknowledgements

The authors were thankful to Drs. Soren K.R. and Senthilkumar M. for providing PCR facilities and ultracentrifugation facilities. The authors also express their gratitude to Director, ICAR-NBAIM, Mau, Dr K. Pandiyan, Dr M. Kumar and Mr. Manish Roy for facilitating SEM micrographs. The authors were thankful to Director, ICAR-IIPR, for co-operation and constant support for executing this work.

Author contributions

SGK conceived, designed and executed the experiments. SP performed various PCRs and trimmed the gene sequences. SGK, SP, SD and JD purified endospore crystal mixture, performed insect bioassay and chitinolytic activity and recorded and digitalized data. SGK and SP prepared SEM samples and biochemical characterization of *Bt* strains. RJ performed nematode bioassay. RJ, SP and JD prepared colloidal chitin. SGK, AC, RJ and SP wrote the manuscript and prepared the graph. SGK, AC and RJ analyzed the experimental data. VK performed protein quantification, protein profiling and interpretation of results.

Funding

The authors did not receive any specific grant from any funding agency for accomplishing this work. The work was accomplished under the institute project entitled "Identification and evaluation of potential *Bt* strains for managing lepidopteran pest of pulse crops" [code: CRSCIIPRSIL201700900148].

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors gave their consent to publish the submitted manuscript as "Original paper" in EJBPC.

Competing interests
The authors declare that the research was conducted in the absence of commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 May 2023 Accepted: 29 July 2023
Published: 8 August 2023

References

Agaisse H, Lereclus D (1995) How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? J Bacteriol 177(21):6027–6032
Ananda MR, Vaiahnav S, Naide PR, Aruna NV (2022) Long term benefits of legume based cropping systems on soil health and productivity. An overview. Int J Environ Clim Chang 12(9):299–315
Anonymous (2022) Directorate of economics and statistics. Department of agriculture and farmers welfare, GOI. https://eands.dacnet.nic.in/Advance_Estimates.htm Accessed on 23rd Oct 2022
Argôlo Filho RC, Gomes RA Jr, Barreto MR, de P Lana UG, Valicente FH, Loguercio LL, (2011) Growth variation among Bacillus thuringiensis strains can affect screening procedures for supernatant-secreted toxins against insect pests. Pest Manag Sci 67(9):1184–1192
Arora N, Ahmad T, Rajagopal R, Bhatnagar RK (2003) A constitutively expressed 36kDa exochitinase from *Bacillus thuringiensis* HD-1. Biochem Biophys Res Commun 307(3):620–625
Asokan R, Swamy HMM, Arora DK (2012) Screening, diversity and partial sequence comparison of vegetative insecticidal protein (vip3A) genes in the local isolates of *Bacillus thuringiensis* Berliner. Curr Microbiol 64(4):365–370
Baragamaarachchi RY, Samarasekera JKRR, Weerasena OVDSJ, Lamour K, Jurat-Fuentes JL (2019) Identification of a native *Bacillus thuringiensis* strain from Sri Lanka active against Dipel-resistant *Plutella xylostella*. PeerJ 7:e7535
Bautista JR, Teves FG (2013) Antibiotic susceptibility testing of isolated *Bacillus thuringiensis* from three soil types around Iligan City. Philipp Afr J Microbiol Res 7(8):678–682
Boonmee K, Thammasittirong SNR, Thammasittirong A (2019) Molecular characterization of lepidopteran-specific toxin genes in *Bacillus thuringiensis* strains from Thailand. Biotech 9(4):1–11
Chak KF, Chao DC, Tseng MY, Kao SS, Tuan SJ, Feng TY (1994) Determination and distribution of cry-type genes of *Bacillus thuringiensis* isolates from Taiwan. Appl Environ Microbiol 60(7):2415–2420
El-kersh TA, Al-sheikh YA, Al-akeel RA, Alsayed AA (2012) Isolation and characterization of native *Bacillus thuringiensis* isolates from Saudi Arabia. Afr J Biotechnol 11(8):1924–1938
Elsayed EA, Othman NZ, Malek R, Awad HM, Wu K, Aziz R, Wadaan MA, El Enshasy HA (2014) Bioprocess development for high cell mass and endospore production by *Bacillus thuringiensis* var *israelensis* in semi-Industrial Scale. J Pure Appl Microbiol 8(4):2773–2783
Engelbrecht G, Horak I, Jansen van Rensburg PJ, Claassens S (2018) Bacillus-based bionematicides: development, modes of action and commercialization. Biocontrol Sci Techn 28:629–653
Franco-Rivera A, Benintende G, Cozzi J, Baizabal-Aguirre VM, Valdez-Alarcón JJ, López-Meza JE (2004) Molecular characterization of *Bacillus thuringiensis* strains from Argentina. Antonie Van Leeuwenhoek 86(1):87–92
Hassan AA, Youssef MA, Elashtokhy MMA, Ismail IM, Aldayel M, Afkar E (2021) Isolation and identification of *Bacillus thuringiensis* strains native of the eastern province of Saudi Arabia. Egypt J Biol Pest Control 31(1):1–11
Hernandez JL (1988) Évaluation de la toxicité de *Bacillus thuringiensis* sur *Spodopterafrugiperda*. Entomophaga 33(2):163–171
Hsu SC, Lockwood J (1975) Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl Microbiol 29(3):422–426
Iatsenko I, Boichenko I, Sommer RJ (2014) *Bacillus thuringiensis* DB27 produces two novel protoxins, Cry21Fa1 and Cry21Ha1, which act synergistically against nematodes. Appl Environ Microbiol 80:3266–3275
Ichikawa M, Uemori A, Yasutake K, Kagoshima K, Mizuki E, Ohba M (2008) Failure to phenotypically discriminate between non-insecticidal *Bacillus thuringiensis* strains with anticancer parasporins (PS2, PS3, and PS4) and *Bacillus thuringiensis* strains that produce insecticidal Cry proteins. Appl Entomol Zool 43(3):421–426
Jisha VN, Benjamin S (2014) Solid-state fermentation for the concomitant production of δ-endotoxin and endospore from *Bacillus thuringiensis* subsp. *kurstaki*. Adv Biosci Biotech 5(10):797
Jones JT, Haegeman A, Danchin EG, Gaur HS, Helder J, Jones MG, Kikuchi T, Manzanilla-López R, Palomares-Rius JE, Wesemael WM, Perry RN (2013) Top 10 plant-parasitic nematodes in molecular plantpathology. Mol Plant Pathol 14:946–961
Lee YK, Jin NY, Lee BR, Seo MJ, Youn YN, Gill SS, Yu YM (2015) Isolation and characterization of new *Bacillus thuringiensis* strains with insecticidal activity to difficult to control lepidopteran pests. J Fac Agric Kyushu Univ 60:103–112
Li H, Bouwer G (2012) Toxicity of *Bacillus thuringiensis* Cry proteins to *Helicoverpa armigera* (*Lepidoptera: Noctuidae*) in South Africa. J Invertebr Pathol 109(1):110–116
Logan NA, Berge O, Bishop AH, Bussse HJ, de Vos P, Fritz D, Kampfer HM, P, Rabinovitch L, Salkinoja-Salonen MS, Seldin L, Ventosa A, (2009) Proposed minimal standards for describing new taxa of aerobic, endospore forming bacteria. Int J Syst Evol Microbiol 59:2114–2121
Lone SA, Malik A, Padaria JC (2017) Selection and characterization of *Bacillus thuringiensis* strains from north western Himalayas toxic against *Helicoverpa armigera*. Microbiology Open 6(6):e00484
Martin PA, Gundersen-Rindal DE, Blackburn MB (2010) Distribution of phenotypes among *Bacillus thuringiensis* strains. Syst Appl Microbiol 33(4):204–208
Masson L, Erlandson M, Puzstai-Carey M, Brousseau R, Juarez-Perez V, Frutos R (1998) A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl Environ Microbiol 64(12):4782–4788
Meena J, Pimentel E (2002) Mechanism of action of *Corynebacterium pauronetabolum* strain C-924 on nematodes. J Nematol 4(2):287
Mendoza G, Portillo A, Arias E, Ribas RM, Olmos J (2012) New combinations of cry genes from *Bacillus thuringiensis* strains isolated from northwestern Mexico. Int Microbiol 15(4):209–216
Mohammed S, El Saedy MA, Enan MR, Ibrahim NE, Ghareeb A, Moustafa SA (2008) Biocontrol efficiency of *Bacillus thuringiensis* toxins against root-knot nematode, *Meloidogyne incognita*. J Mol Cell Biol 7:57–66
Mohan M, Rangeshwaran R, Sivakumar G, Verghese, (2014) Relative toxicity of subspecies of *Bacillus thuringiensis*against lepidopterous insect pests of agriculturalimportance. J Biol Control 28(4):197–203
Murali Krishna T, Devaki K, Swarna B, Abhijit N, Naresh M (2018) Computation of LC50 against *Spodoptera litura* (Fab.) for *Bacillus thuringiensis* isolates from native soil samples of Andhra Pradesh. J pharmacogn phytochem 7(4):174–178
Nair R, Schreinemachers P (2020) Global status and economic importance of mungbean. Mungbean Genome 1–8
Obeidat M, Khyami-Horani H, Al-Momani F (2012) Toxicity of *Bacillus thuringiensis* β-exotoxins and δ-endotoxins to *Drosophila melanogaster*, *Ephestiakuehniella* and human erythrocytes. Afr J Biotechnol 11(46):10504–10512
Ogiwara K, Indrasith LS, Asano S, Hori H (1992) Processing of -endotoxin from *Bacillus thuringiensis subsp. kurstaki* HD-1 and HD-73 by gut juices of various insect larvae. J Invertebr Pathol 60:121–126
Rajashekhar M, Shahanaz E, Vinay K (2017) Biochemical and molecular characterization of *Bacillus* spp. isolated from insects. J Entomol Zool Stud 5(5):581–588

Ramanujam B, Rangeshwaran R, Sivakmar G, Mohan M, Yandigeri MS (2014) Management of insect pests by microorganisms. Proc Indian Natl Sci Acad 80(2):455–471
Roy M, Chatterjee S, Dangar TK (2021) Characterization and mosquitocidal potency of a *Bacillus thuringiensis* strain of rice field soil of Burdwan, West Bengal. India Microb Pathog 158:105093
Sattar S, Biswas PK, Hossain MA, Maiti MK, Sen SK, Basu A (2008) Search for vegetative insecticidal proteins (VIPs) from local isolates of *Bacillus thuringiensis* effective against *lepidopteran* and homopteran insect pests. J Biopestic 1(2):216–222
Sequeros T, Ochieng J, Schreinemachers P, Binagwa PH, Huelgas ZM, Hapsari RT, Juma MO, Kangile JR, Karimi R, Kharirіyatun N, Mbeyagala EK (2021) Mungbean in Southeast Asia and East Africa: varieties, practices and constraints. Agric Food Secur 10(1):1–3
Shikov AE, Malovichko YV, Lobov AA, Belousova ME, Nizhnikov AA, Antonets KS (2021) The distribution of several genomic virulence determinants does not corroborate the established serotyping classification of *Bacillus thuringiensis*. Int J Mol Sci 22(5):2244
Siddiqui IA, Shaukat SS (2003) Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: importance of bacterial secondary metabolite 2,4-diacetylphloroglucinol. Soil Biol Biochem 35:1615–1623
Singh B, Das A, Parihar AK, Bhagawati B, Singh D, Pathak KN, Gupta S (2020) Delineation of genotype-by-Environment interactions for identification and validation of resistant genotypes in mungbean to root-knot nematode (*Meloidogyne incognita*) using GGE biplot. Sci Rep 10(1):1–14
Subbanna ARNS, Khan MS, Srivastava RM, Mishra PK, Babu BK, Venkateswarlu V (2018) Interspecies diversity of *Bacillus thuringiensis* isolates native from North Western Indian Himalayas. J Environ Biol 39(3):306–313
Sujayanand GK, Pandey S, Jagadeeswaran R, Dubey J (2019) Chitinase: a novel biopesticide for insect and nematode pest management. Indian Farmer 6(9):599–603
Sujayanand GK, Akram M, Konda A, Nigam A, Bhat S, Dubey J, Kumar K, Muthusamy SK (2021) Distribution and toxicity of *Bacillus thuringiensis* (Berliner) strains from different crop rhizosphere in Indo-Gangetic plains against polyphagous lepidopteran pests. Int J Trop Insect Sci 1:1–9
Sujayanand GK, Pandey S (2020) Structural and functional diversity of cry genes and their utilization in pest management of grain legumes. In: Microbial mitigation of stress response of food legumes (pp 257–269). CRC Press
Sun YP, Johnson ER (1960) Analysis of joint action of insecticides against houseflies. J Econ Entomol 53:887–892
Tailor R, Tippett J, Gibb G, Pells S, Jordan L, Ely S (1992) Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. Mol Microbiol Berkshire 6(9):1211–1217
Venkataramanamma K, Reddy BV, Jayalakshmi RS, Jayalakshmi V, Rajendran L (2022) Isolation, in vitro evaluation of *Bacillus* spp against Fusarium oxysporum f. sp. ciceris and their growth promotion activity. Egypt J Biol Pest Control 32(1):1–8
Vessey JC, Pegg GF (1973) Autolysis and chitinase production in cultures of *Verticillium albo-atrum*. Trans Br Mycol Soc 60(1):133–143
Vilas-Bôas GT, Lemos MVF (2004) Diversity of cry genes and genetic characterization of *Bacillus thuringiensis* isolated from Brazil. Can J Microbiol 50(8):605–613
Zothansanga R, Senthilkumar N, Gurusubramanian G (2016) Diversity and toxicity of *Bacillus thuringiensis* from shifting cultivation (*jhum*) habitat. Biocontrol Sci 21(2):99–111

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen journal and benefit from:

- ► Convenient online submission
- ► Rigorous peer review
- ► Open access: articles freely available online
- ► High visibility within the field
- ► Retaining the copyright to your article

Submit your next manuscript at ► springeropen.com