



# Toxicity evaluation of Aphidicidal crystalliferous toxins from *Bacillus thuringiensis* strains: a molecular study

Asokan Ramasamy<sup>1\*</sup>, Meghana Suresh<sup>2</sup> and Mahadeva Swamy Hanchipura Mallesh<sup>1\*</sup>

## Abstract

**Purpose:** *Aphis gossypii* and *A. punicae* are the most damaging pests. The emergence of large populations has created concern among farmers because this pest complex is considered critical as it has a significant effect on major crops around the globe. The lack of new technologies for the control of *A. gossypii* and *A. punicae* is also worrying due to the indiscriminate use of chemical insecticides. Besides, this leads to the rapid development of resistance, which strangles their control in the field. Hence, there is a dire need to find the effective biocontrol agent for the management of *Aphis gossypii* and *A. punicae*.

**Methods:** The present investigation emphasizes the isolation and characterization of Aphidicidal *Bacillus thuringiensis* from the Andaman and Nicobar Islands, Karnataka, Assam, Arunachal Pradesh, and Sikkim soil samples. Phase contrast and scanning electron microscopy analysis used to characterize and identify the crystal morphology. Molecular profiling of Bt *cry* genes was determined by PCR using aphidicidal *cry* gene-specific primers, and molecular cloning and sequencing were carried out. Protein profiling by SDS-PAGE analysis was further studied. Finally, a qualitative bioassay of insecticidal activity was carried out against *A. gossypii* and *A. punicae*.

**Results:** A total of 65 *Bacillus*-like colonies were screened; 15 putative Aphidicidal *B. thuringiensis* isolates were identified based on morphological as well as through microbiological studies. Spherical and amorphous crystal inclusion was predominantly present in 34.28% of the *Bt* isolates. Crystal protein profiling of *Bt* isolates by SDS-PAGE analysis showed the presence 130, 73, 34, 25, and 13 kDa bands, among which 50–66 kDa bands were present abundantly. The detection of the *cry* gene of these isolates was done by PCR analysis, which indicated that *cry1*, *cry2A*, *cry3A*, and *cry11A* were on plasmid DNA. All *cry* genes were 80–100% homologous when aligned on alignment tool NCBI-BLASTn and BLASTp. All isolates of *Bt* were tested for their insecticidal activity against aphids. Three of the 15 isolates are Aphidicidal toxin specific by PCR analysis which were observed to be toxic to *Aphis gossypii* and *A. punicae* at a concentration of 35 µg/mL. The observed physical changes were induced by *B. thuringiensis* infection; these strains had been re-isolated from the dead aphids, and the presented results fulfilled Koch's postulates.

**Conclusion:** The present study brought promising *Bt* isolates, primarily capable of creating an efficient biocontrol agent for *Aphis gossypii* and *A. punicae* and various sucking pests soon.

**Keywords:** *Aphis gossypii*, *A. punicae*, Cloning, *cry1*, *cry2A*, *cry3A*, and *cry11A*, Koch's postulates, qualitative bioassay, Sequencing

\* Correspondence: [asokanihr@gmail.com](mailto:asokanihr@gmail.com); [clintonbio@gmail.com](mailto:clintonbio@gmail.com)

<sup>1</sup>Division of Biotechnology, ICAR-Indian Institute of Horticultural Research (IIHR), Hesaraghatta Lake Post, Bengaluru, Karnataka 560089, India  
Full list of author information is available at the end of the article



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## Introduction

Aphids are small sap-sucking insects belong to the insect order Hemiptera, Aphidoidea superfamily (Latreille 1802). Aphid species are major plagues of agricultural, horticultural, and ornamental plants. Aphids damage the crops and reduce farm yields in several ways (Sorensen 2009). An aphid infests extreme damage to leaves and shoots, additionally causing damage to plant growth. They also act as vectors in the transmission of plant viruses (Whitfield et al. 2015). In recent years, the control of aphids with chemical insecticides has become more difficult because aphids have developed resistance to many insecticides (Rousselin et al. 2017). The development of microbial biocontrol agents for aphid use has been typically limited. Currently, more effective means are needed to control the many sap-sucking pests in general and aphids in particular. Advantageously, it would be such an effective means to use specific biological agents. To date, there are no established chemical/biological agents that can effectively control the spread and invasion of plant viruses by aphids, which is why new methods of controlling virus vectors and virus carriers are desperately required. The bacterium *Bacillus thuringiensis* is the most successful insect pathogen used to control insects. This is currently representing about 2% of the total market for insecticides. *Bt* is almost exclusively active toward larval stages of various insect orders and destroys the insect by interrupting the septicemia-accompanied midgut tissue eventually leading to insect death (Raymond et al. 2010). To date, more than 766 *cry* genes have been characterized and grouped from *cry1* to *cry78* based on the amino acid sequence variations (Crickmore et al. 2020; [http://www.lifesci.sussex.ac.uk/home/Neil\\_Crickmore/Bt/toxins2.html](http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html)). *Cry* toxins are generally considered to be less toxic to the environment. It can be integrated easily into biologically regulated pest management systems. Out of *cry78* groups, only *cry2*, *cry3a*, and *cry4* reported active against potato aphid, *Macrosiphum euphorbiae* (Walter and English 1995); *cry4aa*, *cry11aa*, and *cry3a*, reported active against pea aphid (Porcar et al. 2009); *Vip1Ae-Vip2Ae* reported against cotton aphid, *Aphis gossypii* (Sattar et al. 2011).

*Aphis gossypii* Glover (Hemiptera: Aphididae) is a polyphagous insect distributed globally. Host range includes those in Cucurbitaceae, Malvaceae, Solanaceae, and Rutaceae as well as some ornamental plants such as chrysanthemum (Ebert and Cartwright 1997). The aphid causes direct physical damage by extracting carbohydrates and amino acids from plant phloem and also spreads a variety of virus diseases, including *citrus tristeza* virus, watermelon mosaic virus, cucumber mosaic virus, and several potato viruses, resulting in indirect losses to agricultural production. The pomegranate

aphid, *Aphis punicae* Passerini, is distributed throughout the Mediterranean region, the Middle East, Ethiopia, India, Indonesia, Japan, and Pakistan (Bhagat 2012; Blackman and Eastop 2000; Öztürk et al. 2005; Sugimoto 2011) and is a serious insect pest for pomegranate (Mani and Krishnamoorthy 1995; Öztürk et al. 2005; Ananda et al. 2010).

*Aphis gossypii* Glover and *A. punicae* Passerini are devastating pests in agriculture, horticulture, and greenhouse plants. Multiple control strategies, viz biological, chemical, and cultural controls, have proven as a remedy toward these notorious pests. However, the development of insecticidal resistance and the potential threat to human and animal health posed by these chemicals favors biological control. Environmental and human health sustainability requires the discovery of effective biological control strategies and eco-friendly solutions and less use of potentially harmful chemicals. *Bt*-based insecticides are used for controlling lepidopteron (caterpillar), coleopteran (beetle), and dipteran (mosquitoes) insects but are not useful for controlling aphids. Little work were carried out about *Bt* effect on *A. gossypii*. There were no information available on *Bt* effect on *A. punicae*. Therefore, attempts were made to isolate native aphidicidal *Bt* strains with the following objectives: isolation of *Bt*, morphological and microbiological characterization of putative *Bt* strains, molecular characterization of *Bt* strains effective against aphids, cloning of aphidicidal *cry* gene, and bioinformatics research and bioassay against *A. gossypii* and *A. punicae*.

## Materials and methods

### Bacterial strains, plasmids, and media

*B. thuringiensis* subspecies *kurstaki* (HD1) was obtained from the *Bacillus thuringiensis* genetic stock center (BGSC). At the Bio-pesticide Laboratory (BPL), ICAR-Indian Institute of Horticultural Research, Bangalore, native *B. thuringiensis* isolates were chosen from the *Bt* library (Swamy et al. 2011). The *Escherichia coli* strain 105 was used for maintaining the cloned gene.

### Soil sample collection

After gently removing the top debris in the topsoil using a sterile spatula, 100 grams of soil samples are taken from a depth of 5 cm and placed directly inside the sterile polyethylene covers (10 cm × 5 cm). Labels containing the specifics of the date of collection, the place of collection, the name of the collector, and the description of the place of collection where the sampling was performed were written using a permanent marker and placed inside the polyethylene cover and properly secured. Until further use, the soil samples are processed at 4 °C. No commercial formulation of *B. thuringiensis*

was exposed to sampling areas. We tried to collect soil from as different as possible locations.

#### Isolation of *B. thuringiensis* strains

The method of *B. thuringiensis* isolation from various habitats is defined as follows. In a 100-mL conical flask containing 10 mL of Luria Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 1 L of distilled water), buffered with 0.25 M sodium acetate, 1 g of soil sample was suspended. The suspension of the soil was rotated for 4 h at 30 °C in a rotary shaker at 250 rpm. The soil suspension was subsequently heated in a water bath at 80 °C for 10 min to remove the vegetative form of most gram-positive bacteria and total gram-negative bacteria. Serial dilutions ( $10^{-1}$  to  $10^{-4}$ ) in sterile double-distilled water were made after cooling. One hundred microliters of different dilutions were placed on “LB agar” (10 g peptone 140, 5 g yeast extract, 5 g sodium chloride, 12 g agar) and incubated at 30 °C overnight in a bacteriological incubator. For each dilution, there were three replications. After 24 h, flat chalky white or fried egg colonies were picked up and plated on to medium T3 (per liter: 3 g tryptone, 2 g tryptose, 1.5 g yeast extract, 0.05 M phosphate buffer pH 6.8, and 0.005 g of  $MnCl_2$ ) and incubated at 30 °C before lysis. Using single-colony isolation, all selected colonies were purified and spotted on to the master plate.

#### Strain maintenance

Putative Bt Isolates are inoculated into 50 mL of Nutrient Broth and incubated in an incubator at spinning 250 rpm at room temperature. Every culture was periodically examined by phase contrast microscopy until lysed, releasing spores and crystals from the population. Then, 1 mL of aliquot was moved to a cryogenic vial and 20% of glycerol was applied. The suspensions have been vortexed, labeled, and stored at – 80 °C.

#### Scanning electron microscopy (SEM)

Bt strains were grown at 30 °C for 3 days in the T3 medium until sporulation was complete as examined with an oil-immersion lens by a light microscope. The spores and crystals were collected at 4 °C for 10 min at 12,000×g by centrifugation, and the precipitate was washed with ice-cold sterilized double-distilled water three times. After the specimens were air-dried overnight, the spore-crystal suspensions were mounted on the aluminum mount and fixed in 1% osmium tetroxide ( $OsO_4$ ). The samples were then coated with gold in an IB-5 ion coater (LEICA, England); following instructions for the device, SEM observation was performed at a voltage of 15 kV (Zhang et al. 2009).

#### Oligonucleotides and PCR analysis

PCR analysis using universal and gene-specific primers characterized the existence of aphidicidal *cry* genes in native Bt strains. Aphidicidal PCR primers' details are given in Table 1. Total DNA was extracted and purified using the Ferrandis et al. (1999) method described. PCR was performed at a final volume of 50 µl for 250 ng of total *B. thuringiensis* DNA with 2.5 U of Taq DNA polymerase, 200 nM of each dNTP, and 1 mM of each first 3 mM  $MgCl_2$ . Amplification was performed in the thermal cycler of Applied Biosystems under the following conditions: 5 min of denaturation at 94 °C followed by 25 amplification cycles with 1-min denaturation at 94 °C, 45 s of annealing at 45 °C, and 2 min of extension at 72 °C. Upon completion of the 30 cycles, an additional extension phase of 10 min at 72 °C was added. Aphidicidal Bt PCR products are analyzed at 6 V/cm in the TAE buffer (45:1) by 1.5% agarose gel electrophoresis with ethidium bromide (0.5 µg/mL).

#### Molecular cloning of Aphidicidal Bt *cry*-type genes

Using the Fermentas DNA ligation kit (Thermo Scientific, USA), Bt aphidicidal *cry* gene PCR-amplified products were ligated to the TA-cloning vector pTZ57R/T and transformed into *Escherichia coli* DH5  $\alpha$  cells using standard protocols (Sambrook and Russell 2001). The transformed cells (50 µl) were spread to X-gal (270 µg/mL), IPTG (120 µg/mL), and ampicillin (100 µg/mL) containing LB agar plates. The plates were then incubated at 37 °C for blue and white colonies to be screened overnight. Plasmid mobility, colony PCR, and restriction digestion analysis have confirmed cloning. Each *cry* gene clone was sequenced at least three times, and a consensus sequence was obtained. Registered *Cry* sequences have been collected from the National Center for Biotechnology Information's non-redundant protein database (<http://www.ncbi.nlm.nih.gov>).

#### Bioassay

##### Collection of aphids and maintenance of stock culture

The *A. gossypii* samples used in this work were collected from cotton, okra, cucumber, watermelon, and chili pepper cultivated at the Indian Institute of Horticultural Research (IIHR), Bengaluru, India (N 12° 58' E 77° 35') during rabi season 2018–2019, whereas *A. punicae* were gathered from the leaves of pomegranates. *A. gossypii* and *A. punicae* morphological identification was done by Dr. Asokan, IIHR. Live aphids along with the plant material were transferred to the laboratory, where a single adult female apterous parthenogenetic viviparous was used to establish a stock culture of this material in the laboratory and was maintained on respective hosts under glasshouse conditions at 23–25 °C and 15:9 (L to D) photoperiod with 65 ± 5% relative humidity.

**Table 1** Primers used to screen Aphidicidal *cry* genes from native *B. thuringiensis* isolates

Primer	Sequences	Positions	Gene(s) recognized	Product size (bp)	GenBank accession no.	Reference
Set IF	5'-TATGCWCA AGCWGCCAATYTWCATYT- 3'	3 domain blocks 1 and 5	<i>cry1, cry2, cry3, cry4, cry7,</i> and almost all <i>cry</i> genes	-	-	Noguera and Ibarra 2010
Set IR	5'-GGRATAAATTCAATTYKR TCWA-3'					
Set IIF	5'-TTTAGATATTGTTGCA WTATKKYC-3'	3 domain block 2 and 5	<i>cry5, cry8, cry10, cry12, cry13,</i> <i>cry14, cry20, cry40</i> and <i>cry44</i>			
Set IIR	5'-GGRATAAATTCAATTYKR TCWA-3'					
Set IIIF	5'-TATGCWCA AGCWGCCAATYTWCATYT- 3'	3 domain blocks 1 and 4	<i>cry16</i> and <i>cry18</i>			
Set IIIR	5'-CATAACGTAGWY TTAYCTKAWT-3'					
UNcry1(+)	5'-TRACRHTD DBDGTATTAGAT-3'	726	<i>cry1</i>	1500– 1600	-	Seifinejad et al. 2008
UNcry1(-)	5'-MDATYTCTAKRTCTTG ACTA-3'	2268				
SPcry1Aa(+)	5'-TTCCCTTTATTTGGGAAT GC-3'	1023	<i>cry1Aa</i>	1286	M11250	
UNcry1(-)	5'-MDATYTCTAKRTCTTG ACTA-3'	2268				
SPcry1Ab(+)	5'-CGGATGCTCATAGAGG AGAA-3'	940	<i>cry1Ab</i>	1371	M13898	
UNcry1(-)	5'-MDATYTCTAKRTCTTG ACTA-3'	2268				
UNcry2(+)	5'-GTTATTCTTAATGCAGAT GAATGGG-3'	1444	<i>cry2Ab</i>	546	M23724	
SPcry2Ab(-)	5'-TGGCGTTAACAATGGG GGGAGAAAT-3'	1965				
SPcry3A(+)	5'-TGGTGCCCCGTCTAAA CTGAGTGT-3'	180	<i>cry3A</i>	951	U10985	Swamy et al. 2013 (reference Walters and English 1995 and Jouzani et al. 2017)
UNcry3(-)	5'-CATCTGTTGTTTCTGGAG GCAAT-3'	1131				
SPcry4A(+)	ACAGAATCAGCAGTATGG TGG	156–176	<i>cry4A</i>	443	Y00423	Present study (reference Porcar et al. 2009)
SPcry4A(-)	CGCAATCACTAGGATTAG GAGG	577–598				

aB = C, G, or T; D = A or T; H = A, C, or T; K = G or T; M = A or C; R = A or G; Y = T or C

### *B. thuringiensis* toxin preparation

A single colony was suspended in 1 mL of autoclaved water and heated for 15 min at 70 °C to kill vegetative cells, then inoculated in 10 mL of T<sub>3</sub>-sporulation liquid medium (per liter: 3 g of tryptone, 2 g of tryptose, 1.5 g of yeast extract, 0.05 M sodium phosphate [pH 6.8], and 0.005 g MnCl). The culture was grown overnight at 30 °C with continuous shaking at 250 rpm. This 10 mL *Bt* was cultured in 100 mL of T<sub>3</sub>-sporulation liquid medium and incubated for 3 days at 30 °C with continuous shaking at 250 rpm. To monitor external contamination, a regular inspection was performed. Microscopic

observation was also performed to verify the completion of the phase of sporulation. The fully sporulated liquid culture was centrifuged for 10 min at 8000 rpm. Pellets (parasporal crystal proteins and spores) were washed in 20 mL sterile distilled water and centrifuged at 6000 rpm for 30 min. The washing procedure was repeated thrice. The pellets (parasporal crystal protein and spores) were resuspended in 20 mL of sterile distilled water and kept at 4 °C. The protein concentration was estimated using the Bradford method (Sigma-Aldrich, USA) Bradford 1976. Different levels of distilled water have been prepared by diluting. *Bt*'s spore-crystal combination was

then put on a parafilm and coated in a plastic vial with another layer of parafilm where 25 aphids were put (Fig. 1). For either the original toxin-spore suspension or the dilutions, the toxicity of each strain was assessed in triplicate. To avoid physical trauma, the transformation was carefully confirmed to the test cases with a soft brush. Mortality was scored in comparison to parallel control in which aphids fed sterile distilled water instead of bacterial suspension and were used to correct the mortality test.

**Koch's postulates**

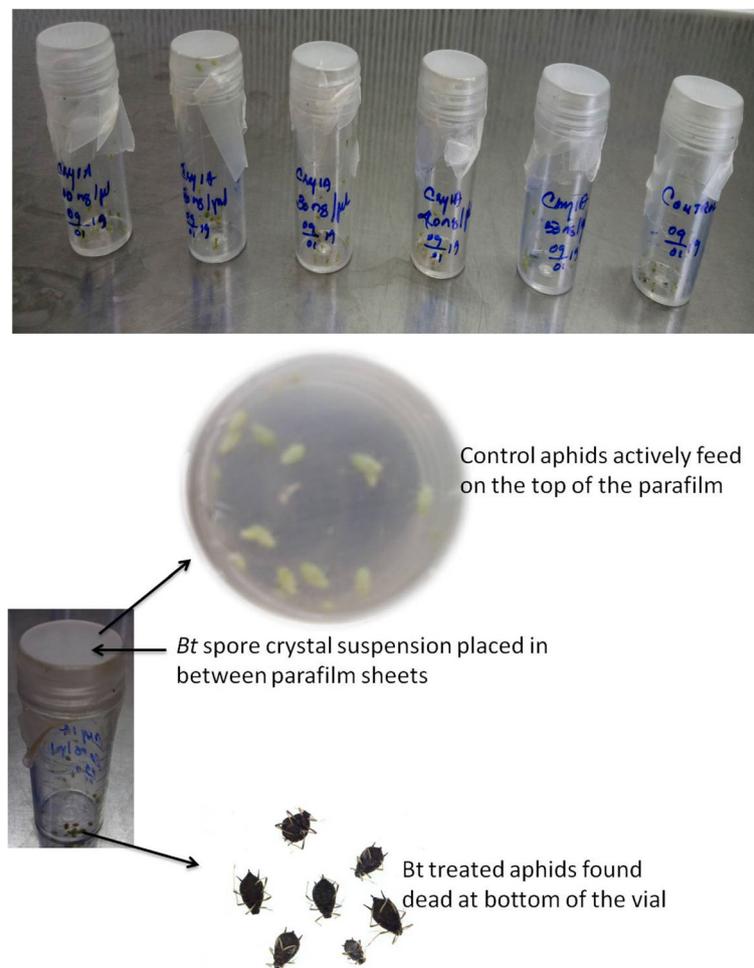
A bioassay resulted in the Bt strains being isolated from dead aphids. From each bioassay, five dead aphids were collected at random. The specimens were individually placed in a 1.5-mL microcentrifuge tube and washed on the surface with 1 mL sterile water. The insects were then placed in a 1.5-mL microcentrifuge tube and homogenized, submerged in double-distilled water, and

incubated for 10 min at 30 °C. A loopful of homogenized insect cells was seeded onto solid nutrient agar (peptic digest of animal tissue 5.0 g, sodium chloride 5.0 g, beef extract 1.5 g, yeast extract 1.5 g, agar 15.0 g, final pH ( at 25 °C)  $7.4 \pm 0.2$ ) in a petri dish and incubated for 12 h. Three individual colonies were taken at random and grown individually in liquid sporulation medium (T3) for 72 h at 30 °C. On solid NA plates, single colonies were restreaked.

**Results**

**Isolation of *Bacillus thuringiensis***

The present investigation conducted experiments with the main objective of obtaining novel aphidicidal isolates of *B. thuringiensis*. It was also intended to understand *B. thuringiensis*' distribution and diversity in different parts of the country. One thousand eighty Bacillus-like isolates are isolated from the Andaman and Nicobar Islands, Karnataka, Assam, Arunachal Pradesh, and Sikkim from



**Fig. 1** Setting up of vials for bioassay Bt spore crystal suspension against aphids. Bioassay of aphids and with Bt crystal suspension in the treatment vial and another vial was given normal water instead of Bt crystal suspension as a control for bioassay

various agro-ecological zones. The isolates were obtained using the standard technique of enrichment defined by Travers et al. 1987. A total of 65 *Bacillus* were screened based on morphological characteristics, and 15 putative *B. thuringiensis* isolates were described. A large number of *B. thuringiensis* are isolated from 87% of samples of rhizospheric soil.

The *B. thuringiensis* index (Bt index) is an important measure of success in isolating *B. thuringiensis*, describing the ratio of *B. thuringiensis* isolates at all locations. The average Bt index for all samples was found to be 0.30 but the index varies by sample and source. *B. thuringiensis* abundance was the highest in all soil samples, with a Karnataka Bt index of 0.66 (Fig. 2, Table 2).

### Colony morphology

Putative Bt as colonies is characterized based on bacterial colony color, texture, elevation, and margin (Fig. 2). The morphological characters showed a wide range of differences, including between strains isolated. According to the Benintende et al. (2001) description, the colonies were medium-sized, whitish, and opaque with irregular edges, a morphology characteristic of *B. thuringiensis*.

### Microscopic characterization of Bt isolates based on crystal morphology

The analysis of the crystal shapes of the 15 isolates performed by a light microscope allowed the sample to be divided into two main classes: 10 isolates producing spherical crystals and 5 isolates producing amorphous and spore adhering crystals. Native putative aphidicidal strains of *B. thuringiensis* develop inclusions of parasporal crystals with different morphologies, sizes, and numbers (Table 3).

### Scanning electron microscopy

SEM analyzed the Bt crystals to demonstrate any further variations between the same crystal shapes of each group. Observations of various *B. thuringiensis* isolates showed clearly the different crystal shapes in the

vegetative and lysis phases (Fig. 3). For the *B. thuringiensis* isolates collected from various environments, the vegetative cells are rod shaped. For all *B. thuringiensis* isolates, the spore form was identical as well. There was a distinction between the different crystal forms such as triangular, bipyramidal, and amorphous, and spore adhering crystals. In the scanning electron microscopy, the crystal forms found in the light microscope were the same.

### Plasmid profiling

Five distinct plasmids were observed in the native Bt isolates in the present study. Megaplasmids are present in low numbers of copies while they are generally present in high numbers of copies as small plasmids. Plasmid patterns are useful tools for characterizing the strains of *B. thuringiensis*. Plasmid patterns are qualitative features, represented by specific plasmid sets, rather than being quantitative features, where differences between patterns can be measured in terms of degrees of similarity.

### Analyses of Aphidicidal cry gene content by PCR

#### Genomic DNA extraction from Bt isolates

PCR analysis will assess insecticidal activity based on the isolates that contain the *cry* gene. Genomic DNA was successfully extracted from the Bt sample; the kit method generally yielded good amounts of DNA and was verified by the use of electrophoresis of the nanodrop and agarose gel. PCR amplification and sequencing of a validated amplicon showed the efficacy of the process. Evaluation of PCR preliminary was done using Noguera and Ibarra's (2010) array of primers which suggests that there were 7 native Bt isolates positive for Array I group of primers. A single molecular weight PCR product was provided by each known Set 1 *cry* gene (Fig. 4). Three indigenous isolates showed similar spore and colony morphology to that of *B. thuringiensis*, whereas no phase contrast microscope was observed. On the other hand, for 2 of them, 3 isolates were positive for the *cry1* genes tested while evaluating PCR. This also refers to the lack of protein-level gene expression. Also,



**Fig. 2** Putative *Bt* like colonies isolated from soil samples collected from the Andaman Nicobar Islands. Laboratory control NA plate and Purified *Bacillus thuringiensis* colony morphology, Bacterial isolate exhibiting the Chalky white with granular periphery a typical *Bt* morphology

**Table 2** Distribution of *B. thuringiensis* and Bt index based on sample and location

Location	Total no. of bacterial colonies	No. of Bacillus colonies	No. of <i>Bt</i> colonies	<i>Bt</i> index
Karnataka	450	23	8	0.66
Andaman and Nicobar Islands	240	16	2	0.34
Assam and Arunachala Pradesh	190	14	2	0.36
Sikkim	200	12	3	0.23
<b>Total</b>	<b>1080</b>	<b>65</b>	<b>15</b>	<b>0.3</b>

in *B. thuringiensis*, crystal protein synthesis is regulated at transcriptional, post-transcriptional, or post-translation levels by a variety of mechanisms (Agaïsse and Lereclus 1995). No combinations of Set 2 or Set 3 Primer generated any desired amplified PCR products.

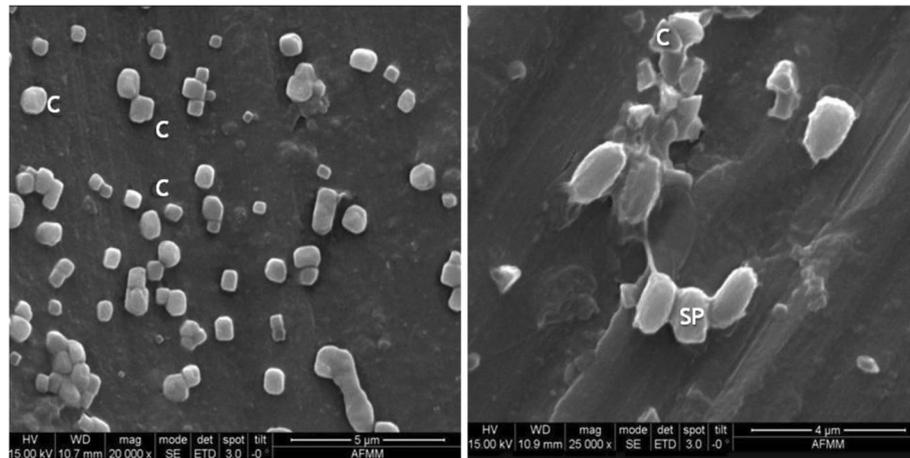
Using *cry1*, *cry2Ab*, *cry3A*, and *cry11A* gene primers, these isolates were further subjected to Aphidicidal *cry* gene analysis. For the *cry1* gene, a total of 8 isolates were amplified (Fig. 5), 2 isolates were amplified for *cry2Ab*, and 3 isolates for *cry3A* gene were amplified.

Aphidicidal *Bt cry* genes are cloned and sequenced successfully. The native *Bt* isolates molecular identification and verifies its identity as *B. thuringiensis* by cloning and

sequencing the Aphidicidal *cry* genes. No pseudogenes and indels have been identified in analyzed nucleotide sequences; in nucleotide NCBI Basic Local Alignment Search Tool search, they fit with high similarity. The sequences of *Bt* Aphidicidal *cry* genes are deposited at the GenBank NCBI. A total of 20 sequences were submitted to the NCBI GenBank database, and accession numbers were obtained. These includes MN101553, MN101554, MN101555, MN101556, MN101557, MN101558, and MN101559 (*cry1*-like genes); MN101560, MN101561, MN101562, MN101563, MN101564, MN101565, MN101566, and MN101567 (*cry1Ab*); MN101568, MN101569, and MN101570 (*cry3a*); and MN101571 and MN101572 (*cry2Ab*).

**Table 3** Colony morphology, vegetative cell type, microscopic studies and crystal shape of native *B. thuringiensis* isolates.

Sl. no.	Isolate name	Colony morphology	Vegetative cell type	Gram's staining	Endospore staining	Crystal staining	Crystal shape
1	BNG_Bta1	Chalky white with granular periphery	Rod shaped	+	+	+	Bipyramidal
2	SHVM_Bta2	Chalky white with granular periphery	Rod shaped	+	+	+	Amorphous
3	MYS_Bta3	Chalky white with granular periphery	Rod shaped	+	+	+	Amorphous and crystals adhere to spore
4	VJP_Bta4	Egg white, medium size, rough, opaque and round	Rod shaped	+	+	+	Spherical
5	DVG_Bta5	White, small, round, bright and runny	Rod shaped	+	+	+	Bipyramidal
6	CMR_Bta6	White, small, round, bright and runny	Rod shaped	+	+	+	Spherical
7	GLB_Bta7	Chalky white with granular periphery	Rod shaped	+	+	+	Spherical
8	BID_Bta8	White, round, runny, larger and less brighter than colony I	Rod shaped	+	+	+	Amorphous and crystals adhere to spore
9	AN_Bta1	White, round, runny, larger and less brighter than colony I	Rod shaped	+	+	+	Small spherical and irregular
10	AN_Bta2	White, small, round, bright and runny	Rod shaped	+	+	+	Amorphous and crystals adhere to spore
11	AS_Bta1	Chalky white with granular periphery	Rod shaped	+	+	+	Small spherical and irregular
12	As_Bta2	White, round, runny, larger and less brighter than colony I	Rod shaped	+	+	+	Spherical
13	SK_Bta1	Egg white, medium size, rough, opaque and round	Rod shaped	+	+	+	Amorphous and crystals adhere to spore
14	Sk_Bta2	White, small, round, bright and runny	Rod shaped	+	+	+	Spherical
15	SK_Bta3	Chalky white with granular periphery	Rod shaped	+	+	+	Small spherical and irregular



**Fig. 3** Scanning electron micrographs of *Bacillus thuringiensis* spores and crystals (20000 x). C-Cuboidal; Sp- Spore; C-Amorphous

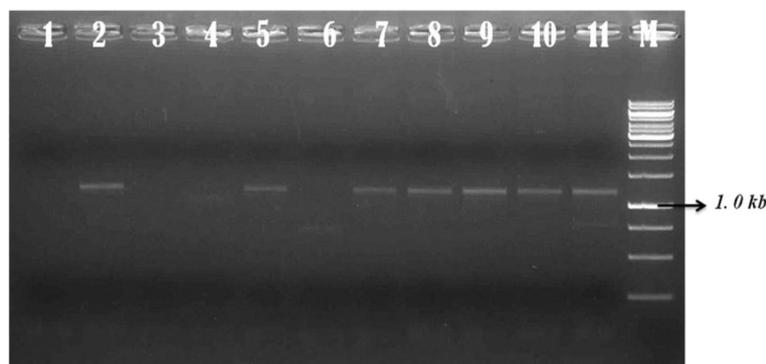
#### Crystal protein profiles of *B. thuringiensis* isolates

SDS-PAGE is widely used to compare *B. thuringiensis* isolate protein profiles. SDS-PAGE studied isolates containing Aphidicidal-specific *cry* genes presenting crystals to estimate the molecular weight of the Cry proteins. Isolates had molecular weight  $\delta$ -endotoxins between 13 and 130 kDa, but the most typical pattern consisted of molecular weight proteins between 50 and 60 kDa. Isolates showed different bands from 1 to 9 (Fig. 6).

#### Bioassay/toxicity of Cry toxins against aphids

All fifteen new/novel Cry-related proteins displayed aphidicidal activity against the *Aphis gossypii* and *A. punicae* aphids along with reference strain HD-1 in two different forms: pre-solubilized (spore crystal complex) and solubilized (pro-toxin form). Mortality was higher

than 90% with the two concentrations used (10 and 100  $\mu\text{g}/\text{mL}$ ) in the preliminary bioassays. The screening was performed against adults of *Aphis gossypii* and *A. punicae* aphids in a controlled environment of  $18 \pm 2^\circ\text{C}$ ,  $70 \pm 10\%$  RH, and 16:8 L to D. The  $\text{LC}_{50}$  value was estimated using four concentrations, 100, 200, 300, 400, and 500  $\mu\text{g}/\text{mL}$  that killed  $18\% \pm 6\%$ ,  $25\% \pm 5\%$ ,  $58\% \pm 7\%$ , and  $85\% \pm 4\%$ , respectively, of the aphids. The Cry-related protein's concentration-mortality responses for *Aphis gossypii* and *A. punicae* match a line of regression. The interaction between the host population and  $\log$  [protein dose] was not significant with the protein, exhibiting a median lethal concentration ( $\text{LC}_{50}$ ) of 35  $\mu\text{g}/\text{mL}$ . Bioassay studies have shown greater efficacy in the pre-solubilized form (92% mortality) and fewer efficacies in a solubilized form (45%). For 25 aphids per replication, the bioassay repeated three times. All the treated aphids



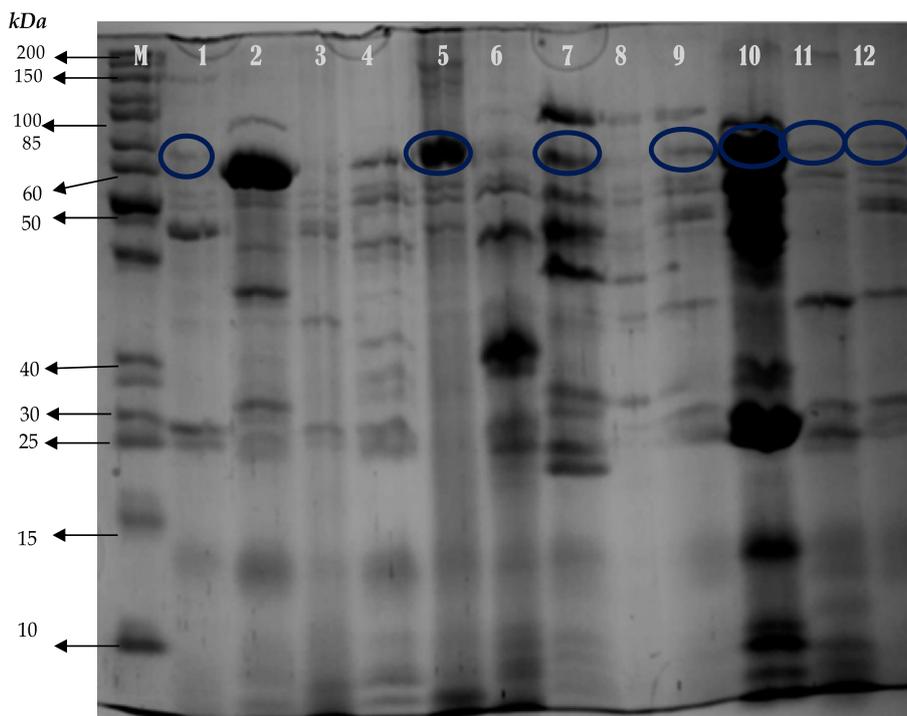
**Fig. 4** The 1.5% ethidium bromide-stained agarose gel showing PCR amplification from the selected native *B. thuringiensis* stains using Set1 primer combinations. Legends: M—Molecular Weight Marker (1.0 kb, Thermo Scientific, USA), Lane1—BNG\_Bta1, Lane2—SHVM\_Bta2, Lane3—MYS\_Bta3, Lane4—VJP\_Bta4, Lane5—DVG\_Bta5, Lane6—CMR\_Bta6, Lane7—GLB\_Bta7, Lane8—BID\_Bta8, Lane 9—AN\_Bta1, Lane10—AN\_Bta2, Lane 11—AS\_Bta1



**Fig. 5** The 1.5% ethidium bromide-stained agarose gel showing PCR amplification from the selected native *B. thuringiensis* stains using *cry1Ab* gene primer combinations. *Legends:* M—Molecular Weight Marker (100 bp, Thermo Scientific, USA), Lane1—BNG\_Bta1, Lane2—SHVM\_Bta2, Lane3—MYS\_Bta3, Lane4—VJP\_Bta4, Lane5—no Amplification, Lane6—CMR\_Bta6, Lane7—no amplification, Lane8—BID\_Bta8, Lane 9—AN\_Bta1, Lane10—AN\_Bta2, Lane11–13—no amplification, Lane 14—genomic negative control (without genomic DNA), Lane15—water control (Water served as a template instead of DNA).

were dead by the end of the toxicity test. All the 25 aphids in controls were found to be safe at the end of bioassay studies. A Tukey test was conducted, and no significant differences between the strains were found ( $\alpha = 0.005$ ). But a significant difference between strains and untreated aphids has been observed. Three native Bt strains are found to be most toxic compared to the standard reference strain (Table 4) from this study. The untreated adult *Aphis gossypii* and *A. punicae* controls

remained in steady growth, continued feeding, and during the first 48 h showed no color change, after 60 h (Fig. 7). In adults exposed to spore crystal suspensions in the *Aphis gossypii* and *A. punicae*, disease signs were variable with each strain, but the following general pattern of symptoms (Fig. 7) was observed. Amid the initial 24 h, the *Aphis gossypii* and *A. punicae* adults were moving and encouraging and did not hint any contamination. After 48 to 60 h, adults of *Aphis gossypii* and *A.*



**Fig. 6** Sodium dedocyle sulfate polyacrylamide gel electrophoretic pattern of total proteins of sporulated Bt. The lanes from left to right show protein pattern of native Bt aphidicidal Isolates. M—PageRuler™ Unstained Protein Ladder (10 to 200 kDa), Lane1—BNG\_Bta1, Lane2—SHVM\_Bta2, Lane3—MYS\_Bta3, Lane4—VJP\_Bta4, Lane5—DVG\_Bta5, Lane6—CMR\_Bta6, Lane7—GLB\_Bta7, Lane8—BID\_Bta8, Lane9—AN\_Bta1, Lane10—AN\_Bta2, Lane11—AS\_Bta1 and Lane12—Sk\_Bta2

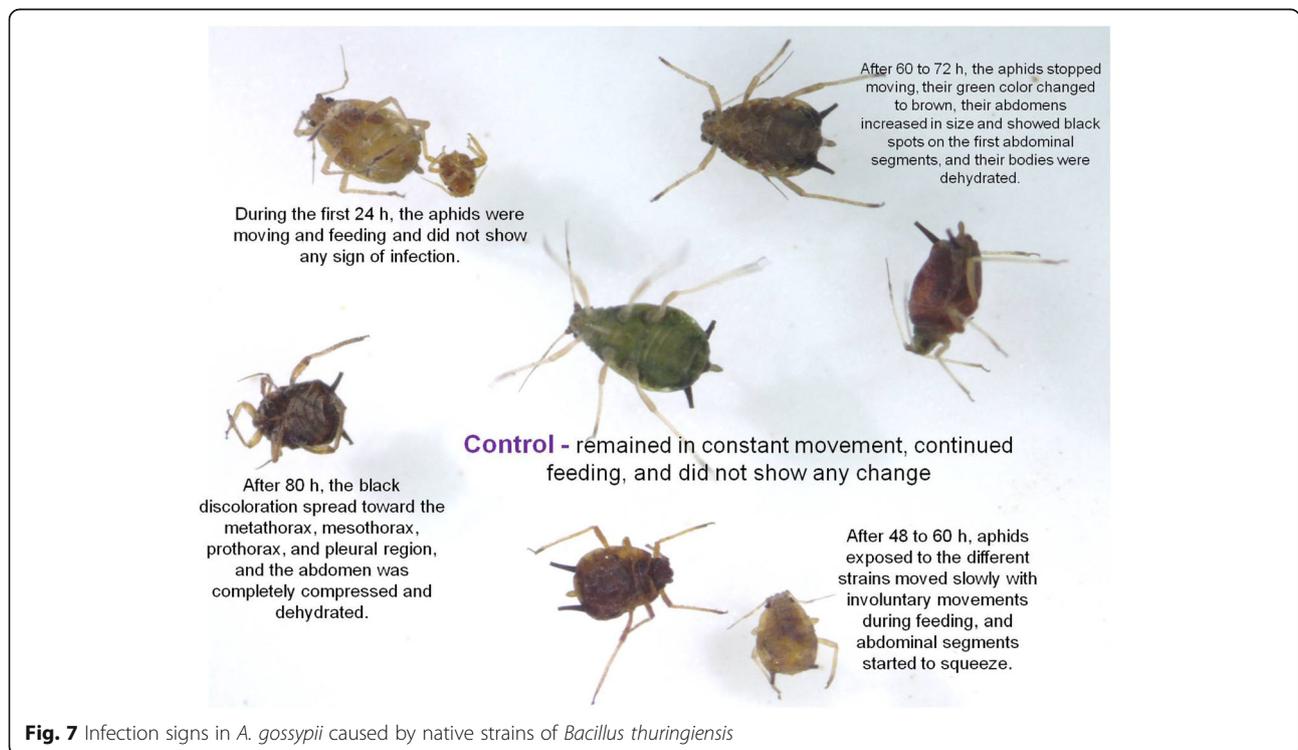
**Table 4** Insecticidal activity of the native *B. thuringiensis* isolates against *A. gossypii* and *A. punicae* adults

Sl. no.	<i>Bt</i> isolates	Mortality (%) <sup>a</sup> <i>A. gossypii</i>	Mortality (%) <sup>a</sup> <i>A. punicae</i>	LC <sub>50</sub> <sup>b</sup>	Standard error		95% Confidence intervals <sup>c</sup>		Goodness of fit			P value
					Bottom	Top	Bottom	Top	R square	Absolute sum of squares	Sy.x	
1	BNG_Bta1	90	92	380.8	1.586	1.586	20.17 to 26.94	7.396 to 14.16	0.6838	339.8	4.759	< 0.0001
2	SHVM_Bta2	91	89	380.9	1.586	1.586	20.17 to 26.95	7.396 to 14.17	0.6838	339.8	4.759	
3	MYS_Bta3	91	96	380.1	1.586	1.586	20.17 to 26.96	7.396 to 14.18	0.6838	339.8	4.759	
4	VJP_Bta4	75.55	72	372.7	1.244	1.244	15.68 to 20.98	5.460 to 10.76	0.6924	208.9	3.732	
5	DVG_Bta5	91	91	380.1	1.586	1.586	20.17 to 26.96	7.396 to 14.18	0.6838	339.8	4.759	
6	CMR_Bta6	82.22	80.60	279.4	1.101	1.556	17.99 to 22.68	3.349 to 9.984	0.7741	218	3.812	
7	AN_Bta1	90	89.9	380.8	1.586	1.586	20.17 to 26.94	7.396 to 14.16	0.6838	339.8	4.759	
8	AN_Bta2	77.77	77.75	342.1	1.062	1.062	17.74 to 22.26	5.181 to 9.708	0.8233	152.2	3.186	
9	AS_Bta1	90	91	380.8	1.586	1.586	20.17 to 26.94	7.396 to 14.16	0.6838	339.8	4.759	
10	As_Bta2	84.44	85	372.7	1.47	1.47	17.09 to 23.36	4.422 to 10.69	0.7122	291.8	4.41	
11	SK_Bta1	80	79	350	1.246	1.246	16.57 to 21.88	6.011 to 11.32	0.7052	209.6	3.738	
12	Sk_Bta2	80	82	350.1	1.246	1.246	16.57 to 21.89	6.011 to 11.33	0.7052	209.6	3.738	
13	<b>SK_Bta3</b>	<b>90</b>	<b>92</b>	<b>380.8</b>	<b>1.586</b>	<b>1.586</b>	<b>20.17 to 26.94</b>	<b>7.396 to 14.16</b>	<b>0.6838</b>	<b>339.8</b>	<b>4.759</b>	
14	<b>GLB_Bta7</b>	<b>92.22</b>	<b>91.20</b>	<b>336.7</b>	<b>1.299</b>	<b>1.299</b>	<b>21.34 to 26.88</b>	<b>8.342 to 13.88</b>	<b>0.7695</b>	<b>227.8</b>	<b>3.897</b>	
15	<b>BID_Bta8</b>	<b>93.33</b>	<b>94.0</b>	<b>322.2</b>	<b>1.596</b>	<b>1.596</b>	<b>19.49 to 26.29</b>	<b>7.488 to 14.29</b>	<b>0.6534</b>	<b>343.8</b>	<b>4.787</b>	
16	<i>Bt kurstaki</i> (HD1)	88.88	89.60	372.2	1.549	1.549	17.03 to 23.64	6.365 to 12.97	0.6124	324	4.648	

<sup>a</sup>Results are expressed as nanograms of toxin per square centimeter of surface

<sup>b</sup>Fl<sub>95</sub> min.–max., 95% confidence limit

<sup>c</sup>The highest mortality (%) observed in the bioassays (at 300 ng toxin/cm<sup>2</sup>)



**Fig. 7** Infection signs in *A. gossypii* caused by native strains of *Bacillus thuringiensis*

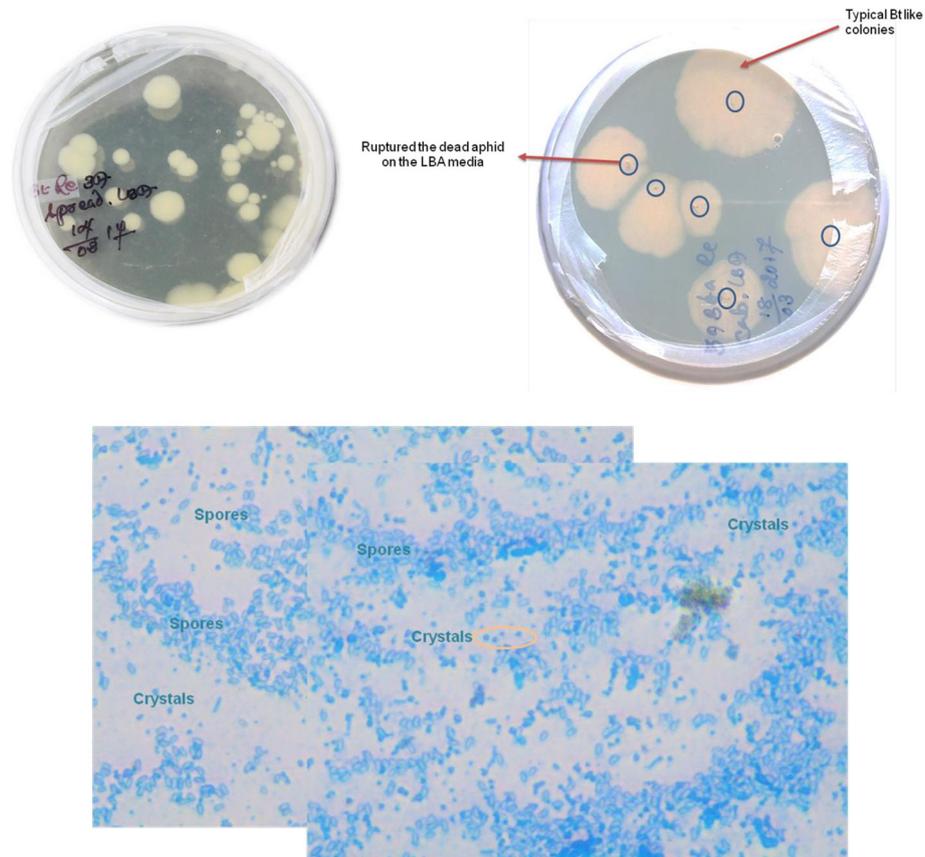
*punicae* exposed to various strains moved slowly during feeding with involuntary movements and abdominal segments started to collapse. The Bt-treated *Aphis gossypii* and *A. punicae* adults stopped moving after 60 to 72 h, their color changed to brown, their abdomen increased in size and displayed black spots on the first abdominal segments, and their bodies were dehydrated (Fig. 7). The dark staining extended toward the thorax after 80 h, and the abdomen was packed and dried out completely (Fig. 7).

#### Koch's postulates

In the present study, Koch's postulates were tested in aphids carcass with native *B. thuringiensis* strains. The results of Koch's postulate suggest that Bt strains were responsible for the death of the exposed *Aphis gossypii* and *A. punicae* and that the physical changes observed were caused by Bt infection; these strains were re-isolated from the dead insects, and the results reported satisfied Koch's postulates (Fig. 8).

#### Discussion

*Aphis gossypii* Glover (Homoptera: Aphididae) harbors a broad range of host plants (Fuller et al. 1999). Under favorable conditions, *A. gossypii* can produce up to 60 generations/year. Depending on the infestation rate and crop stage, crop production may be reduced by up to 90% by *A. gossypii*. This aphid can attack from the seedling stage, reproduce through the parthenogenesis of the telitoc, and grow in 5–7 days to adults. These reproductive characteristics favor the rapid selection of resistant individuals when populations are exposed to environmental stress factors like insecticides (Liu et al. 2005). *A. punicae* Passerini (Homoptera: Aphididae) is one of the key pests in pomegranate. They are well known for their ability to decrease plant vigor, facilitate mold growth on leaves, and therefore reduce crop quality and yield. Both adults and nymphs feed on fruit, leaves, and inflorescences (Moawad and Al-Barty 2011). Extensive use of pesticides to control aphids has led to resistance to the major classes of insecticides, such as organophosphates, carbamates, and pyrethroids (Jansen 2000). As a result,



**Fig. 8** Aphidicidal *Bt* strains were re-isolated from the dead aphids to fulfil Koch's postulate. A typical white and opaque and round colony morphology was prominent. Crystal staining of *Bt* re-isolated from dead *A. gossypii* and *A. punicae*

safe alternative strategies for controlling these aphid species are required. The aim of the present study was, therefore, to investigate the pathogenicity of *B. thuringiensis* against aphids, taking into account the pests and their significances. *B. thuringiensis* subspecies currently account for approximately 98% of formulated sprayable microbial pesticides (Lacey et al. 2015). However, it still accounts for just 2% of the global pesticide market (Bravo et al. 2011). Since very few but known Bt strains are active against Hemipterans, it is suggested that consideration should be given to the characterization of native Bt isolates for insecticidal activity against aphids. It allows other researchers to concentrate on isolating native strains from different environments. It is very important to find out the novel Bt. Native novel/new Bt strains of greater toxicity, particularly in transgenic Bt crops or bio formulations, to assist and address the issue of insect resistance.

Bt diversity is being sought worldwide by researchers, giving more bio-insecticidal choices and alternatives to address insect resistance. Bt has been isolated from various ecologies since its first Ishiwata isolation in 1902 and has been widely studied. Despite this exploration around the world, research on this bacterium is still incomplete (Melo et al. 2016). The bacterial concentration mainly depends on the type of environmental sample. The soil is the primary source of Bt isolates (Delucca et al. 1982). As it was collected from 70% of the soil samples in the world (Martin and Travers 1989), the current research disclosed that the soil is indeed the best habitat for the isolation of *B. thuringiensis* regardless of geographical places; the isolates were acquired, indicating they are widespread. When used for confinement, some of the soils witnessed no Bt in them. The first level of grouping was done by colony morphology (Fig. 2 and Table 2). The colonies of putative Bt isolates were medium-sized, whitish, and opaque with irregular edges, a morphology characteristic of *B. thuringiensis*, as Benintende et al. (2001) describes it. The next qualitative analysis of Bt isolates was done by light microscopy. Ten isolates produce spherical crystals, and five isolates show amorphous and spore adhering crystals. This was contrary to the usual environmental sample of Bt screenings, where most produce bipyramidal crystals (Meadows et al. 1992; Bernhard et al. 1997). Scanning electron microscopy was used in this work not only to confirm the shape of the crystal but also to further magnify and differentiate between the shapes of the spherical crystal. Although there were previous reports of different types of spherical crystals (Noguera and Ibarra 2010), it was rarely used as a tool for classifying large Bt collections (Djenane et al. 2017). From SEM images, it was found that there are four kinds of spherical forms in the collection among the spherical crystals. This illustrates the wide diversity between the Bt strains.

Parasporal crystals are encoded by plasmid genes, with an equal number of plasmid patterns expected. But, contrary to the published work and theories on Bt parasporal crystals (Aptosoglou et al. 1997; Reyes-Ramírez and Ibarra 2008; Fagundes et al. 2011), among the collection isolates, only five plasmid patterns were identified. PCR amplifications were carried out with universal and aphidicidal gene-specific primers, to predict their insecticidal activity. The majority of 8 isolates were amplified for *cryI* gene, 2 isolates amplified for *cry2Ab*, and 3 isolates amplified for *cry 3A* gene. Aphidicidal *cry* gene amplification, cloning, sequencing, and comparison with published strains from the NCBI database confirmed the identity of the isolates as Bt strains. All of the isolates belong to the Bt family. Bt isolates harbor *cryI*, *cry3a*, and *cry2Ab* genes. PCR is a highly sensitive and relatively rapid technique and is especially suitable for fast, large scale, first-tier screening of Bt isolates. Bt strains harboring novel *cry* genes and also, the less frequently observed *cry* genes, have been identified by PCR, using specially designed primers corresponding to the highly conserved regions (Bravo et al. 1998; Kim et al. 1998, Porcar and Juarez-Perez 2003; Wang et al. 2003). In the present investigation, Aphidicidal *cry* genes were successfully cloned and sequenced. By cloning and sequencing the Aphidicidal *cry* genes, the native Bt isolates molecular identification confirmed its identity as *B. thuringiensis*. No pseudogenes and indels were found in analyzed nucleotide sequences; they match with high similarity in nucleotide NCBI Basic Local Alignment Search Tool search. The Bt Aphidicidal *cry* genes sequences were deposited at NCBI GenBank.

SDS-PAGE assessment of crystal protein profiling of Bt isolates showed the existence of bands 130, 73, 34, 25, and 13 kDa, among which there were abundantly 50–66 kDa bands. Even the protein in pre-solubilized and solubilized form varying from 13 to 130 KDa has been discovered to be toxic to *Aphis gossypii* and *A. punicae*, insects requiring further investigation. The Bt strain protein profiles were differentiated by Haggag and Yousef (2010) into three primary groups, group I (28–58 KDa), group II (60–80 KDa), and group II (125–150 KDa). The majority of indigenous isolates included the three groups as a whole.

Wellman-Desbiens and Côté (2004) reported the toxicity of *B. thuringiensis* against sucker insects in an experiment performed on stinkbug nymphs *Lygus hesperus* (Hemiptera: Miridae), treated with *B. thuringiensis* grown on artificial diet. After 7 days of the assay, the strains tested reached about 98% mortality against *L. hesperus*. Melatti et al. (2010) established a methodology for selective bioassay of *B. thuringiensis* against *A. gossypii*. The bioassay with four hundred strains of *B. thuringiensis* showed that the strains S29, S40, S616, S1168,

and S1576 had the highest toxicity to *A. gossypii*, causing mortality rates above 50% on average and exhibiting the highest potential for pest control. Among them were the most efficient strains S29 and S1168, which caused deaths of 76 and 73%, respectively, against *A. gossypii*. Sap-sucking insect pests (aphids, whiteflies, and bugs of plants) showed very low susceptibility to certain Cry proteins (Chougule et al. 2012) with the lowest reported  $LC_{50}$  values ranging from 70 to 100  $\mu\text{g}/\text{mL}$  for Cry4Aa and higher than 500  $\mu\text{g}/\text{mL}$  for Cry3A and Cry11Aa against the pea aphid *Acyrtosiphon pisum* (Harris) (Hemiptera: Aphididae) (Porcar et al., 2009). Multiple variables may be responsible for the low toxicity of Cry proteins against hemipteran pests with lepidopteran pests, the most significant being the intestinal pH that is acidic in aphids and alkaline in lepidopterans and the abundance and formation of proteolytic enzymes, cysteine proteases in aphids, and primarily serine proteases (trypsin and chymotrypsin) in lepidopterans. This explains the higher toxicity of trypsin-pre-activated Cry3A, Cry4A, and Cry11A against *A. pisum* (Porcar et al., 2009). Palma et al. (2014) successfully identified novel cry-related genes from Bt strain H1.5, cloned and expressed in *E. coli*. The insecticidal activity of the purified recombinant protein was tested in bioassays against several lepidopteran pest species and the green-peach aphid *M. persicae*. This novel Cry-related protein showed the lowest  $LC_{50}$  value (32.7  $\mu\text{g}/\text{mL}$ ) reported to date for a Cry protein against a hemipteran pest. The toxicity assessment of Bt on *Aphis gossypii* and *A. punicae* was studied in this research, showing that the native Bt isolates were toxic. According to our results, three of the *B. thuringiensis* strains tested were able to cause death to *Aphis gossypii* and *A. punicae*. The mortality rate observed in the control treatment was always 0%, allowing to infer that the mortality observed was due to the strains of *B. thuringiensis* and indicating that the method is reliable for the initial strain selection. The thorough bioassay against distinct species of aphids with distinct levels and diverse circumstances should be carried out in the future.

## Conclusion

This study isolates and characterizes the active Aphidicidal cry genes from native Bt isolates. The genetic characterization of the collection offers the possibility of selecting the strain to be tested against sucking pests in general and especially aphids and other agricultural insect pests in bioassays. In quantitative data, the effectiveness of *B. thuringiensis* Cry proteins against *A. punicae* is practically unavailable. Current research has focused on the development of a sound insect sucking pest management scheme with a strong focus on *Aphis gossypii* and *A. punicae* aphids. Bioassay results suggest that the

*Bt* toxins were responsible for the mortality of the exposed aphids, and the observed physical changes were induced by *B. thuringiensis* infection; these strains had been re-isolated from the dead aphids, and the presented results fulfilled Koch's postulates. Accordingly, indigenous Bt strains can provide high aphid mortality and can be used as an essential component of integrated pest management. Additional studies are needed to screen and develop a bioinsecticide that can be used systemically to control *A. gossypii* and *A. punicae*.

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## Authors' contributions

MSHM and MS designed the experiments and carried out all experiments. MSHM and AR wrote and revised this manuscript. The authors read and approved the final manuscript.

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## Ethics approval and consent to participate

This article does not contain any studies performed with animals, and no human testing was involved.

## Competing interests

Authors declare that they have no competing interests.

## Author details

<sup>1</sup>Division of Biotechnology, ICAR-Indian Institute of Horticultural Research (IIHR), Hesaraghatta Lake Post, Bengaluru, Karnataka 560089, India.

<sup>2</sup>Department of Biotechnology, University of Agricultural Sciences (UASB), Gandhi Krishi Vignan Kendra (GKVK), Bengaluru, Karnataka 560 065, India.

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