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Characterization of lepidopteran-specific *cry1* and *cry2* gene harbouring native *Bacillus thuringiensis* isolates toxic against *Helicoverpa armigera*

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ABSTRACT

Bacillus thuringiensis (*Bt*) based biopesticides are feasible alternatives to chemical pesticides. Here, we present the distribution of lepidopteran-specific *cry1* and *cry2* genes in native *B. thuringiensis*. Forty four out of 86 colonies were found to harbour crystals by phase contrast microscopy exhibiting a *Bt* index of 0.51. PCR analysis resulted in the amplification of *cry1* in 24 and *cry2* in 14 isolates. Twelve of the isolates showed presence of both *cry1* and *cry2*, while 18 isolates did not show presence of either of the genes. Toxicity screening using spore-crystal mixtures against 2nd instar larvae of *Helicoverpa armigera* revealed that the isolates (50%) were either mildly toxic or not toxic (36.36%), and only 13.63% were toxic. The results are interesting, particularly so because the same isolates were previously reported to contain lepidopteran specific *vip3A* genes also, hence can complement the toxicity of the isolates harbouring *vip3A* genes.

1. Introduction

As the world's population is increasing geometrically, achieving global food security-making safe and nutritious food accessible to everyone, and achieving so sustainably is a challenging task. Feeding estimated 9.2 billion people in 2050 would require raising overall food production by about 70% [1]. A major bottleneck in achieving this challenge is the competition from the insect pests. Insect pests are responsible for destroying one fifth of the world's total crop production annually, leading to heavy economic losses. The major damaging insect pests of crops belong to the order Lepidoptera [2] and *Helicoverpa armigera* is one of the most significant lepidopteran pests with potential to attack more than 180 species of plants [3]. It is widely distributed in Asia, Europe, Africa and Australasia causing damages worth 2 billion US dollars annually, excluding the socio-economic and environmental costs associated with the use of chemical insecticides and the introduction of GM crops [4–6]. *H. armigera* has over the years developed resistance to various chemical insecticides [7,8] and of late, its resistance to genetically modified crops expressing insecticidal protein from *B. thuringiensis* has also been reported [9,10].

The most common method to control insect pest populations is the use of chemical insecticides. Two of their properties, long residual action and toxicity to a wide spectrum of organisms made chemical insecticides very useful against insect pests. However, extended use of certain chemical insecticides have caused many environmental problems like persistence, toxicity to non-target organisms including humans and development of insect resistance [11,12]; reviewed in [13]. One of the most promising alternatives to the man-made chemical pesticides is the use of natural insect pathogen, *Bacillus thuringiensis* (*Bt*). The entomopathogenic potential of *Bt* is primarily due to its ability to produce insecticidal crystalline proteins (Cry and Cyt) [14] and in certain cases due to the production of vegetative insecticidal proteins (Vips) [15]. The crystalline and vegetative insecticidal proteins are respectively produced during the sporulation and vegetative stages of *Bt*growth. Upto November 2016, seventy four classes of Cry proteins (Cry1-Cry74), three classes of Cyt proteins (Cyt1-Cyt3) and four classes of Vip proteins (Vip1-Vip4) have been designated based on their amino acid sequence homology [16]. These toxins are highly specific in action, harmless to humans and other vertebrates and are biodegradable. Presently, there are more than 50,000 known strains of *B. thuringiensis* isolated from diverse environments around the world [17,18]. These strains exhibit varying degree of toxicity against different pests. Despite the availability of such large collection of *B. thuringiensis* strains and their insecticidal genes, three events have rendered the search for novel insecticidal strains/genes more urgent. First, a significant number of pests are not controlled with the available Cry proteins. Second, at times the level of expressed toxins is not high enough to kill the host and third, after many years of successful use in the field, the first cases of resistance to *B. thuringiensis* have appeared [19].

Jammu and Kashmir (32°00′–36°10′ North and 73°22′–77°40′ East)

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falls in the great North-Western complex of the Himalayan ranges having complex geomorphology. The variations in topographical features along longitude, latitude and altitude of the region create climatic variations resulting in unique and rich biodiversity [20], thereby making this North-Western Himalayan region a critical biodiversity hotspot of the world. These distinctive features and diversity of insects in the region provide an opportunity for prospecting novel *B. thuringiensis* strains with novel combinations of crystalline protein coding genes having wide insecticidal spectrum. The ecological distribution of this bacterium in Jammu and Kashmir region remains largely unexplored. The aim of this study was to isolate *B. thuringiensis* strains from Jammu and Kashmir region and to assess their geographical diversity with respect to the presence of lepidopteran-specific (*cry1* and *cry2*) genes content to assess their toxicity against *H. armigera*.

2. Materials and methods

2.1. Sample collection and isolation of Bacillus thuringiensis

A total of 86 isolates were analysed in this study which were obtained from various soil samples, collected from forests, lake sediments and agricultural fields of Kashmir valley in our previous study [21]. Sites with no history of *Bt* application were selected (Table 1). Isolation of *Bt* was done by enrichment using acetate selection as described by Travers et al. [22]. Briefly, 1 g of soil samples were incubated in 10 ml of LB broth buffered with sodium acetate solution (0.3 M, pH 6.8) at 30 °C for 4 h. In order to eliminate non-sporulated microbes that germinated during incubation, 2 ml aliquot of each sample was heated at 80 °C for 10 min. The surviving spores were diluted (10–1000 folds) in sodium acetate buffer (pH 6.8) from which 300 µl of each was spread on T3 agar plates and incubated at 30 °C to grow for 72 h. For each plated sample, well isolated colonies representing *Bacillus* like morphology were picked and purified on T3 agar plates containing penicillin at a concentration of 10 $\mu g ml^{-1}$.

2.2. Scanning electron microscopy

Following acetate selection, isolates that tested positive for growth on T3 agar medium amended with penicillin at a concentration of 10 $\mu g ml^{-1}$ were examined for the presence of parasporal crystals [23,24]. The different stages of bacterial growth as well as the crystal production was analysed by Scanning Electron Microscopy (SEM) as described previously [21]. *Bt* index was calculated as described by Baig et al. [25].

Table 1
Features of sampling sites, success of *Bt* isolation and the distribution of *cry1* and *cry2* genes in the native isolates.

Soil Type	Total No. of colonies	No. of *Bacillus* like[a] isolates	No. of Bt isolates	Bt index[b]	*cry* genes present	
					cry1%/no.	*cry2*%/no.
Forest	513	45	27	0.60	55.55/15	37.03/10
Lake sediment	18	8	6	0.75	66.66/4	00.00/0
Agricultural (Maize field)	609	33	11	0.33	45.45/5	36.36/4
Total	1140	86	44	0.51	54.54/24	31.81/14

[a] Off-white, opaque, slightly raised, and regular outlined.
[b] *Bt* Index: *Bacillus thuringiensis* isolation index was calculated by dividing the number of *Bt* isolates by the total number of *Bacillus* like colonies obtained.

2.3. 16S rRNA gene sequencing

Total cell DNA was extracted from the native and reference strains using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) as per the manufacturer's instructions. PCR amplification of 16S rRNA gene from the selected *Bacillus* isolates was performed using the universal primers: forward (27f) 5′-AGAGTTTGATCCTGGCTCAG-3′, reverse (5210r) 5′-AAGGAGCTGATCCAGCCGCA-3′. The purified amplicons of 16S rRNA gene were sequenced using primers 27f and 5210r with fluorescent terminators (Big Dye, Applied Bio systems). The identity of the sequences obtained were determined by NCBI BLAST [26].

2.4. PCR amplification of cry1 and cry2 genes

Using total DNA as template, the *cry* genes were amplified using primers (+)5′-MDATYTCTAKRTCTTGACTA-3' and (−)5′-TRACRHTDDBDGTATTAGAT-3′ [27] for the detection of *cry1* and (+) 5′-GTTATTCTTAATGCAGATG-AATGGG-3′ and (−)5′-CGGATAAAAT-AATCTGGGAAATAGT-3′ [28] for the detection of *cry2* class of genes. PCR was performed in an Eppendorf Mastercycler and amplification conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 47 °C and 59 °C for *cry1* and *cry2* primers, respectively for 60 s, and extension at 72 °C for 60 to 90 s (depending on the expected size of amplicon), and a final extension step at 72 °C for 10 min. An aliquot of the samples were mixed with 6X DNA loading buffer so that the final concentration of the mixture is 1X, electrophoresis was carried out at 70–80 mA current in 1X TAE, for 45–90 min. An appropriate DNA marker was run alongside in one of the lanes. To visualize the DNA bands gels were viewed under UV light (λ = 254 nm) and images captured using AlphaImager HP (ProteinSimple, USA) gel documentation system.

2.5. Insect bioassays

The insecticidal activity of *cry* gene harbouring *Bt* isolates was evaluated against 2nd instar larvae of *H. armigera* by "quick screen" method [29]. The spore crystal mixtures were obtained as described for microscopy analysis. Higher doses of spore crystal mixtures (1 $mg ml^{-1}$) were smeared on young chick pea leaves in triplicates. The larvae were initially left for 10 h with the leaves smeared with spore crystal mixtures and then allowed to feed on fresh leaves for 12 h. Based on their ability to subsequently feed on the fresh diet, the treated larvae were scored as: not eaten was assigned a score of 3; partially eaten a score of 2 and completely eaten a score of 1. Based on the scores assigned, the toxicity potential of the isolates was classified as, non-toxic for isolates displaying score of 1.0, uncertain toxicity for isolates with score > 1.0 but < 2.0 and toxic for isolates exhibiting score of ≥2.0. *B. thuringiensis* subsp. *kurstaki* HD-1 was used as positive control and solubilisation buffer as negative control. The conditions for bioassay were as; 25 °C, 50 ± 10% RH and a 14:10 (light/dark [h]) photoperiod.

3. Results

3.1. Identification of B. thuringiensis

Eighty six *Bacillus* isolates were recovered by applying acetate-penicillin selection methodology to 30 soil samples collected from three types of soils (Table 1). Forty four isolates were found to synthesize crystal protein during sporulation by phase contrast microscopy. The cell shape of *B. thuringiensis* was recorded at different stages of growth (Fig. 1). *B. thuringiensis* was successfully isolated from all types of samples, recovery was highest from lake sediments (0.75) followed by forest soils (0.60) and the least from agricultural soils (0.33) (Table 1). The average *Bt* index of the study was 0.51 (Table 1). A 1500 bp amplicon corresponding to 16S rRNA was successfully amplified in all the

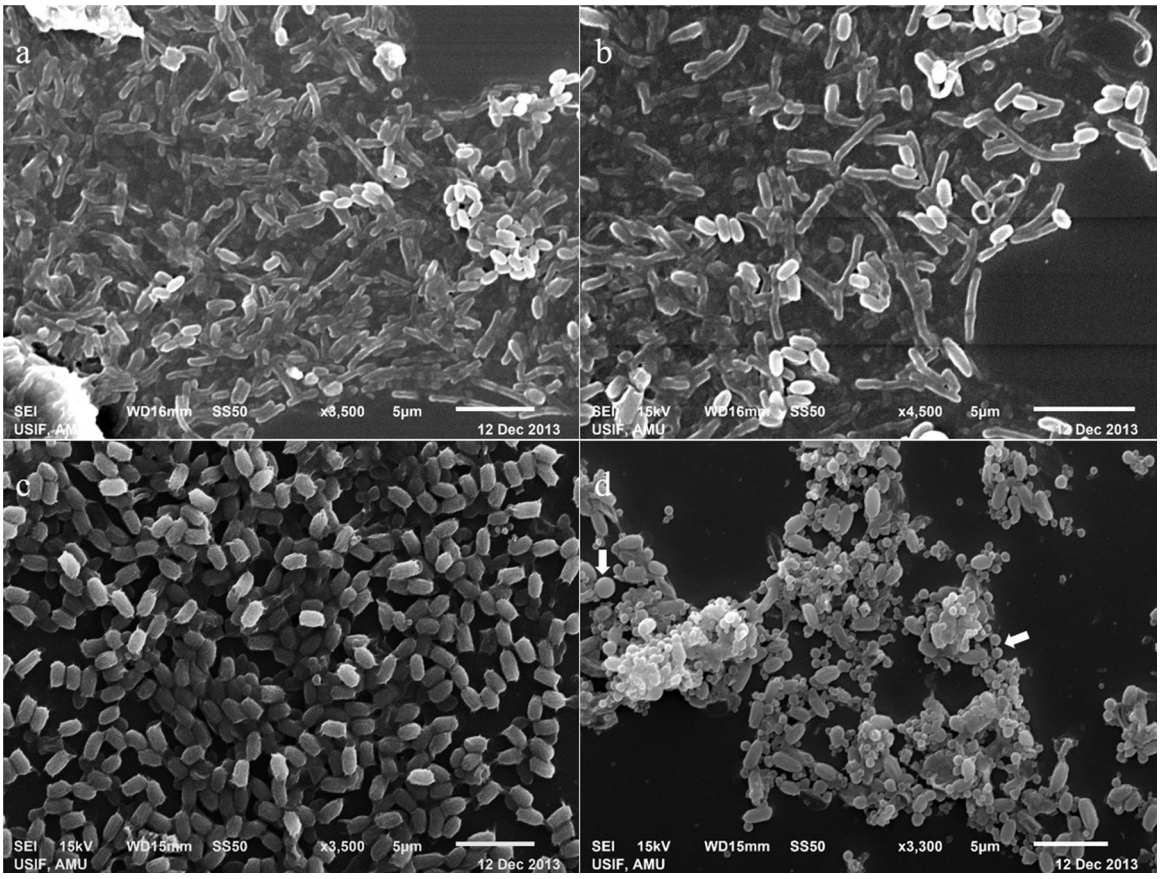

Fig. 1. Scanning electron micrographs of *B. thuringiensis* cells showing different stages of growth. (a) log phase (16 h), (b) late log phase (20 h), (c) sporulation phase (48 h), (d) late sporulation/crystal production (72 h), white arrows indicate the spherical crystals.

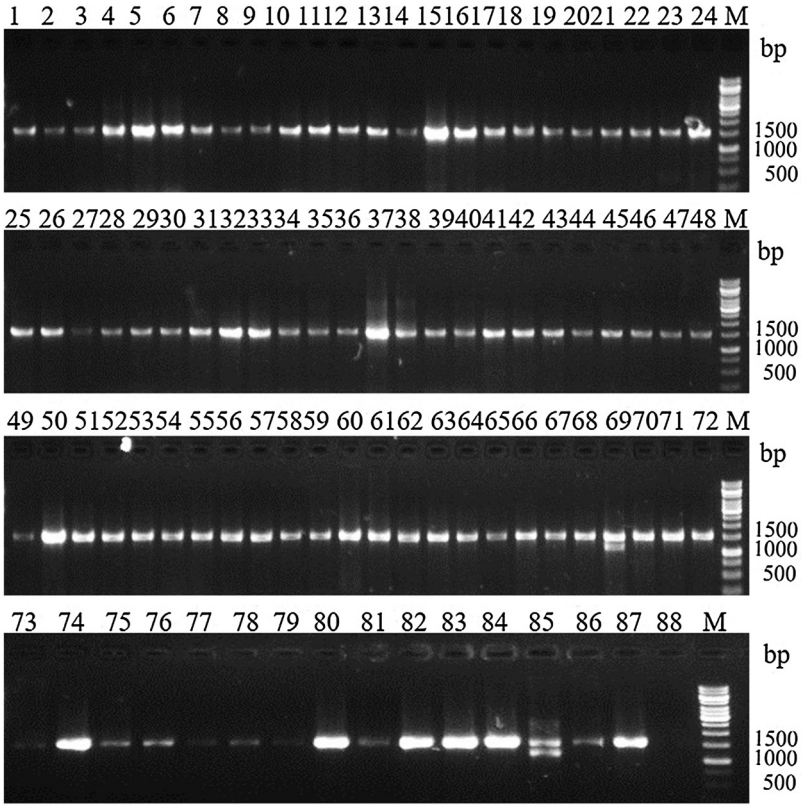

Fig. 2. PCR amplification of 16S rRNA gene of the native *Bacillus* isolates (Lanes 1–86 native isolates, 87, positive control (*Bt* subsp. *kurstaki* HD1 template), 88, negative control (without template). Lane M: 1 kb DNA size marker (Fermentas), 1.0% agarose/EtBr gel.

Table 2
Phylogenetic identification and *cry* gene profiling of native isolates obtained from various locations of Kashmir Valley.

Isolates	Isolation site	Accession No.	Nearest Phylogenetic Neighbor	Identity (%)	*cry* gene profile[d]
JK7	Brarinar (Forest soil)	KJ125312	KF631232 *Bacillus thuringiensis* strain MU9	100	I, II
JK9	Brarinar (Forest soil)	KJ125313	KF512665 *Bacillus thuringiensis* strain BFB35	99	I, II
JK10	Brarinar (Forest soil)	KJ125314	KF550441 *Bacillus thuringiensis* strain WBD10A	100	NA
JK11	Brarinar (Forest soil)	KJ125315	KF555624 *Bacillus thuringiensis* strain Dr45	99	NA
JK12	Brarinar (Forest soil)	KJ125316	FR877760 *Bacillus thuringiensis* strain BD12	100	I, II
JK13	Brarinar (Forest soil)	KJ125317	KF444375 *Bacillus thuringiensis* strain WG47(1)	100	NA
JK14	Brarinar (Forest soil)	KJ125318	JX500188 *Bacillus thuringiensis* strain EAPL17	100	I
JK15	Brarinar (Forest soil)	KJ125319	KF550441 *Bacillus thuringiensis* strain WBD10A	99	I, II
JK16	Brarinar (Forest soil)	KJ125320	KF550441 *Bacillus thuringiensis* strain WBD10A	99	I, II
JK17	Brarinar (Forest soil)	KJ125321	KF500576 *Bacillus thuringiensis* strain *Bt*100	99	NA
JK18	Brarinar (Forest soil)	KJ125322	KC778385 *Bacillus thuringiensis* strain BGB20	100	I
JK19	Brarinar (Forest soil)	KJ125323	KF010790 *Bacillus thuringiensis* strain B45V	100	I, II
JK20	Pangkong (Lake Sediment)	KJ125324	JQ004442 *Bacillus thuringiensis* strain GTG-47	99	NA
JK21	Pangkong (Lake Sediment)	KJ125325	JQ004442 *Bacillus thuringiensis* strain GTG 47	99	I
JK22	Pangkong (Lake Sediment)	KJ125326	EU240956 *Bacillus thuringiensis* strain DW-1T	98	I
JK23	Pangkong (Lake Sediment)	KJ125327	KF017270 *Bacillus thuringiensis* strain VITGS	99	NA
JK26	Pangkong (Lake Sediment)	KJ125330	KF010790 *Bacillus thuringiensis* strain B45V	100	I
JK27	Pangkong (Lake Sediment)	KJ125331	KF631232 *Bacillus thuringiensis* strain MU9	99	I
JK33	Kalgi (Maize Field)	KJ125337	KF550441 *Bacillus thuringiensis* strain WBD10A	100	I, II
JK35	Kalgi (Maize Field)	KJ125338	JQ004425 *Bacillus thuringiensis* strain GTG-21	99	NA
JK36	Kalgi (Maize Field)	KJ125339	KF017270 *Bacillus thuringiensis* strain VITGS	99	II
JK37	Kalgi (Maize Field)	KJ125340	JQ004442 *Bacillus thuringiensis* strain GTG-47	98	I
JK38	Kalgi (Maize Field)	KJ125341	KF017270 *Bacillus thuringiensis* strain VITGS	99	I
JK39	Kalgi (Maize Field)	KJ125342	KF631232 *Bacillus thuringiensis* strain MU9	99	NA
JK40	Kokarnag (Forest soil)	KJ125343	EF495116 *Bacillus thuringiensis* strain 19198	95	I
JK41	Kokarnag (Forest soil)	KJ125344	JQ004425 *Bacillus thuringiensis* strain GTG-21	100	NA
JK42	Kokarnag (Forest soil)	KJ125345	EF113653 *Bacillus thuringiensis* strain GDFT2	99	NA
JK52	Mairan (Maize field)	KJ125355	EU429663 *Bacillus thuringiensis* serovar *kurstaki*	96	NA
JK57	Gulmarg (Forest soil)	KJ125358	KF555624 *Bacillus thuringiensis* strain Dr45	98	NA
JK58	Gulmarg (Forest soil)	KJ125359	KF444375 *Bacillus thuringiensis* strain WG47(1)	99	I, II
JK59	Gulmarg (Forest soil)	KJ125360	KF550441 *Bacillus thuringiensis* strain WBD10A	99	NA
JK60	Gulmarg (Forest soil)	KJ125361	KF631232 *Bacillus thuringiensis* strain MU9	99	NA
JK65	Gulmarg (Forest soil)	KJ125362	KF017270 *Bacillus thuringiensis* strain VITGS	99	I, II
JK66	Gulmarg (Forest soil)	KJ125363	JQ004425 *Bacillus thuringiensis* strain GTG-21	99	I, II
JK67	Gulmarg (Forest soil)	KJ125364	KF017270 *Bacillus thuringiensis* strain VITGS	99	I
JK70	Venkara (Forest soil)	KJ125366	KF512665 *Bacillus thuringiensis* strain BFB35	100	NA
JK71	Venkara (Forest soil)	KJ125367	JX051373 *Bacillus thuringiensis* strain T106	98	NA
JK72	Venkara (Forest soil)	KJ125368	JX437002 *Bacillus thuringiensis* strain RX-MKV2	99	I, II
JK73	Venkara (Forest soil)	KJ125369	GQ497139 *Bacillus thuringiensis* strain KKK 2	98	I
JK74	Venkara (Forest soil)	KJ125370	KF631232 *Bacillus thuringiensis* strain MU9	99	NA
JK75	Saripara (Maize Field)	KJ125371	JQ004425 *Bacillus thuringiensis* strain GTG-21	99	II
JK76	Saripara (Maize Field)	KJ125372	KF631232 *Bacillus thuringiensis* strain MU9	99	NA
JK88	Udusa (Maize Field)	KJ125383	KF631232 *Bacillus thuringiensis* strain MU9	99	I
JK92	Udusa (Maize Field)	KJ125387	JQ004442 *Bacillus thuringiensis* strain GTG-47	99	I, II

isolates using total cell DNA as template (Fig. 2). The amplicons were sequenced and the partial sequences were aligned with the 16S rRNA gene sequences of *Bt* available from GenBank. It was observed that the obtained sequences showed 95–100% identity with the reported sequences on BLAST analysis (Table 2). The partial 16S rRNA gene sequences were submitted to GenBank and accession numbers were obtained [21].

3.2. Amplification of cry genes

PCR screening using two pairs of universal primers was performed to detect *cry1* and *cry2* gene families in our collection. Primers for the detection of both *cry1* and *cr2* showed successful amplification as indicated by their specific product sizes of 1500 and 700 bp, respectively on agarose gel electrophoresis (Fig. 3). Twenty six isolates of the collection were found to contain either or both the *cry* genes tested while as 18 isolates did not show the presence of either of the genes. Compared to *cry2*, *cry1* genes were found to be more abundant and in most of the isolates (12) they were found together.

3.3. "Quick screen" and toxicity assay against Helicoverpa

All the 44 native *B. thuringiensis* isolates and the reference strain HD1 were subject to toxicity screening using concentrated spore crystal suspensions (1 $mgml^{-1}$). Twenty two out of 44 isolates were assigned a score of 1.0-2.0 as the leaves were partially eaten, 16 isolates were assigned a score of 1.0 because the leaves were completely eaten in this case while as only six isolates and the reference strain were assigned a score of 2 as the leaves were not eaten at all by the larvae. The scores indicate the mild toxicity, absence of toxicity and high toxicity of the isolates, respectively (Fig. 4).

4. Discussion

Although, distribution of *cry* genes of *B. thuringiensis* in some geographical regions of India has been investigated earlier [30,31], no researcher has systematically analysed the distribution of *cry* genes in soils of Jammu and Kashmir region. So, it is of interest to determine the distribution and diversity of *cry* genes and identify the type of *cry* genes. Since lepidopteran pests are the most predominant among all other insect pests and cause heavy economic loss, this study was carried to characterize and study the distribution of *Bt* isolates containing lepidopteran-specific *cry1* and *cry2* genes. Further, the toxicity of the isolates was evaluated against an economically important lepidopteran pest *H. armigera*.

The presence of a parasporal inclusion body is a diagnostic feature to discriminate *B. thuringiensis* species form its close relatives in the *Bacillus cereus* group [32]. Based on the presence of parasporal crystals,

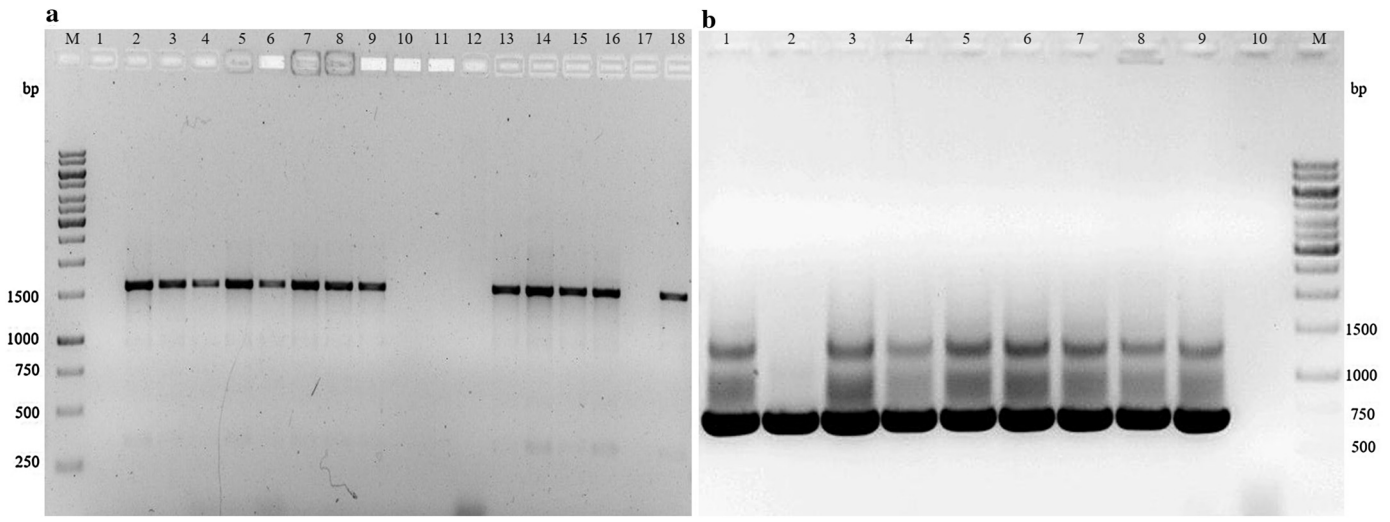

Fig. 3. Amplification of (a) *cry1* and (b) *cry2* genes in representative *B. thuringiensis* isolates indicating amplification of 1500 bp and 700 bp amplicons, respectively. Lane M: 1 kb DNA size marker (Fermentas). 1.0% agarose/EtBr gel.

44 *Bacilli* out of 86 were classified as *B. thuringiensis*. *B. thuringiensis* was found to be widely distributed in the soils of Jammu and Kashmir region with no history of *Bt* application. The estimation of the success of *Bt* isolation (*Bt* index) varied among the soil samples and the average *Bt* index observed in the study was 0.51. The average *Bt* index varies between soil samples all over the world as reported previously [25,21,33,34]. The likely reason for difference in *Bt* index is hard to access, however, the difference in geomorphology and the interaction of the bacterium with their insect hosts could influence their numbers in different environments.

16S rRNA gene sequencing is a routine method used for the taxonomic identification of bacteria [35]. However, the method has not been successful in clearly distinguishing the members of the *B. cereus* group of which *B. thuringiensis* is one of the member [35]. So, the partial 16S rRNA gene sequences were considered *B. thuringiensis* for the statistical counting if they showed maximum similarity to either *B. thuringiensis* or *B. cereus*.

PCR-based approach has been extensively utilized for the identification of known and novel *cry* genes in *B. thuringiensis* since its introduction by Carozzi et al. [36]. The rapidity and reproducibility of PCR-based detection of *cry* genes makes it a very useful tool even today. Both *cry1* and *cry2* genes were detected in the isolates, *cry1* was found to be more prevalent than *cry2* and most of the *cry1* harbouring isolates also contained *cry2* gene. The predominance of *cry1* gene compared to all other *cry* genes in the native *B. thuringiensis* has been reported extensively [37,38]. However, predominance of *cry* genes other than *cry1* has also been reported [25,28,39,40]. Twenty six out of 44 *B. thuringiensis* isolates showed the presence of either *cry1* or *cry2* genes among which 12 isolates showed the presence of *cry1* and *cry2* in combination (Table 2). The possible reason for both *cry1* and *cry2* genes being found together is that both the genes may be closely located on the genome and have evolved together [41]. Isolates containing both the *cry* genes are ideal for development of biocontrol agent as both the proteins encoded by these genes are specific against lepidopteran pests. Eighteen of the 44 isolates did not show presence of either *cry1* or *cry2*, suggesting that they may contain other types of *cry* genes not tested in this study. A large number of isolates harbouring no *cry* genes has been reported in various *B. thuringiensis* collections previously [42,43].

All the putative *B. thuringiensis* isolates were subjected to toxicity screening against 2nd instar larvae of *H. armigera* using higher doses of spore crystal mixtures. Only 13.63% of the isolates exhibited toxicity as evidenced by the treated larvae not being able to feed on fresh leaves, 50% of the isolates were mildly toxic whereas 36.36% were found to be non-toxic (Fig. 4). The difference in toxicity is multifactorial including the absence of toxin proteins and due the poor expression of the Cry proteins.

In the present study, *B. thuringiensis* isolates from various soil samples were found to harbour either or both of the *cry1* and *cry2* genes and some of them were found to be toxic against *H. armigera*. More precise bioassays using different doses of spore crystal mixtures against more number of larvae shall lead to the identification of potentially toxic isolates for the control of *H. armigera*.

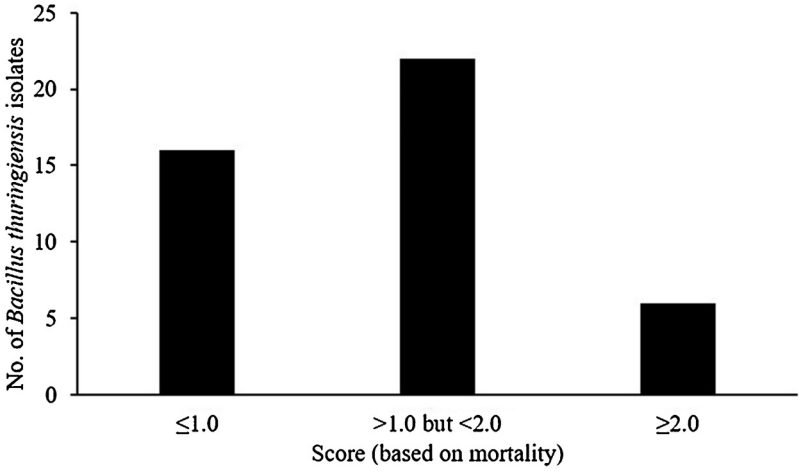

Fig. 4. Toxicity distribution of *B. thuringiensis* isolates against *H. armigera* larvae. The values indicate ≤1.0, non-toxic; > 1.0 but < 2.0, mildly toxic; ≥2.0, toxic.

Conflict of interest

The authors declare no conflict of interest.

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