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Isolation and Identification of Native *Bacillus thuringiensis* Isolates

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A B S T R A C T

The use of insect pathogens is an alternative for insect control because of their specificity and least impact on the environment. The identification of new and highly potent strains of *Bacillus thuringiensis* has become inevitable to combat insect resistance. To search for new *Bacillus thuringiensis* isolates with novel insecticidal properties against the most devastating Lepidopteran insect pests specifically *Helicoverpa armigera*, 400 samples were collected from different regions of the 8 states in India. Of 874 Bt like bacteria obtained using the acetate selection method 150 were identified as *Bacillus thuringiensis*. Microscopic observation of the spore–crystal mixture of 150 isolates revealed 6 different types of crystal proteins. Spherical crystals were predominantly present in 72% of the total *Bacillus thuringiensis* isolates. In this study, unique oval insecticidal crystal proteins were observed in 4.66% of the Bt isolates. The SDS-PAGE analysis of the insecticidal crystal proteins with different shapes and sizes showed protein bands in the range of 45–130 kDa. This confirms the great diversity with respect to the presence of insecticidal crystal proteins. The isolates need to be screened for the presence of lepidopteran toxic insecticidal crystal proteins encoding *cry1* and *cry2* genes to explore their potential application in insect pest control.

***Key words*:** *Bacillus thuringiensis, Cry* genes, Insecticidal crystal proteins (ICP)

Many insect pests of the order Lepidoptera cause frequent and serious damage to agriculturally important crops and frequently lead to significant yield loss to farmers and the national economy. The use of chemical insecticides to control the insect pests without following recommended insect pest management (IPM) practices, leads to the emergence of resistance in insects and environmental degradation [1]. Microbial insecticides are an alternative to chemical insecticides with insect specificity and safety; therefore, they are used in integrated pest management. Professor S. Ishiwata the Japanese scientist, first isolated Bt from diseased silkworms in 1901 [2]. Bt is a ubiquitous rod shaped, gram-positive, spore forming bacterium. During sporulation, it produces parasporal crystalline inclusions called insecticidal crystal proteins (ICP) or δ-endotoxin. These proteins are toxic to insect larvae in the orders Lepidoptera, Diptera and Coleoptera [3]; but they are harmless to most of the other organisms, including wildlife and beneficial insects [4]. When the crystal proteins are ingested by insects, they are solubilized in the midgut, forming toxins. The toxicity of these crystals to the insects is determined by the presence of the specific receptors in the midgut epithelium [5]. Spores and parasporal crystal proteins produced by Bt have been used to control insect pests since the 1920s and are often applied as sprays [6] and are used under the trade names such as DiPel and Thuricide. Several collections of Bt isolates have been well characterized worldwide. The commercially available Bt strains have been isolated from various habitats such as soil, plant leaves, dead insects, and stored grain, as well as aquatic environments such as marine sediments, mangroves, and freshwater [7]. Diverse formulations containing bacterial agents such as suspension concentrate formulation of *Bacillus thuringiensis* var. *kurstaki* are used for effective management of *Helicoverpa armigera* [8], *Spodoptera litura* [9], *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* have been developed for mosquito control [10]. As insects develop resistance to Bt, new strains of are isolated, tested and introduced over time [11]. The *cry* genes encoding the crystal proteins were among the first to be used in the genetic engineering of plants for enhanced insect resistance [12]. The identification of *cry* genes in the Bt isolates is done using polymerase chain reaction (PCR). Moreover, the reliability of the insecticidal activity of the identified *cry* genes is purely dependent on their expression. A thorough characterization of Bt strains should be done with the determination of parasporal crystal composition and toxicity by bioassays [13].

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For the last few decades, researchers have been intensely working on collecting and analyzing novel Bt strains. The main objective of this study is to screen the soils collected from diverse habitats of Indian geography. The variations in geographical features along latitude, longitude, and altitude of the regions create climatic variations resulting in unique and rich biodiversity [14], thereby making this region a critical biodiversity hotspot. These distinctive features and diversity of insects in the region provide an opportunity for mining novel *Bt* strains with novel combinations of ICP coding genes having wide insecticidal spectrum. The ecological distribution of this bacterium in Indian soils remains largely unexplored. The aim of this study was to isolate *B. thuringiensis* strains from Indian soils and to assess their geographical diversity with respect to the presence of lepidopteran-specific (*cry1* and *cry2*) genes content. Different methods have been used to identify Bt strains and characterize the isolates by microscopic studies, colony morphology, and ICP profiling by SDS-PAGE.

MATERIALS AND METHODS

The soil samples were collected from diverse undisturbed fields, including the agricultural, non-agricultural lands of Andhra Pradesh, Gujarat, Haryana, Himachal Pradesh, Maharashtra, Rajasthan, Tamil Nadu, and Uttar Pradesh in India. Approximately 5 g of each soil sample was collected 5 cm below the soil surface by scraping off the surface material with a sterile spatula. These samples were stored in sterile zip lock bags at ambient temperature till analysis is carried out.

Selective isolation of Bt from soil samples

A modified method based on acetate selection [15] was used to screen the soil samples to detect and identify Bt. Approximately 1.0 g of each sample was suspended in 10 mL LB - sodium acetate medium [LB broth (Himedia) with 2M sodium acetate, pH 6.8), vortexed vigorously and incubated overnight at 30°C in a rotary shaker at 150 rpm. The suspension was pasteurized for 10 min at 80°C in a water bath to kill the non-spore forming bacteria. An aliquot of 1 mL of pasteurized sample was serially diluted up to 10^{-8} dilution. An aliquot of 200 µL each from 10^{-6} to 10^{-8} dilutions was spread on T3 agar (3 g Tryptone, 2 g Tryptose, 1.5 g Yeast Extract, 0.05 M di and mono basic Sodium phosphate salts (pH 6.8), 0.005 g $MnCl_2$ per liter). Traverse *et al*. [15] and incubated at 30°C for 48 h. From the T_3 agar plates, colonies resembling Bt were selected and sub-cultured for further purification and analysis.

Characterization of isolates for colony and ICP morphology

Colony morphology was studied in single colonies developed on T_3 agar. Colonies that were off-white to cream in colour with circular to irregular margins were selected. For microscopic studies, the isolates were inoculated into 5 mL of T_3 broth and incubated at 30°C with 200 rpm for 2–8 days, and the bacterial sporulation was monitored through a light microscope. A loop full of lysed culture of the selected isolates was made into a smear on a glass slide and heat-fixed. The heat-fixed smear was stained with a few drops of Coomassie Brilliant Blue stain (0.133% Coomassie Brilliant Blue G250 in 50% acetic acid) for 1 min. The stained smears were washed and observed under light microscope for the presence of crystalline inclusions [16]. The Btk HD-1 strain was used as a reference strain for microscopy. The isolates having visible parasporal crystals (ICPs) were identified as Bt and were inoculated into 10 mL of T3 broth and incubated at 30°C overnight with 180 rpm shaking conditions. An aliquot of 1 mL of overnight culture was transferred into 1.5 mL sterile vials containing 20% glycerol and stored at -80°C as stock cultures for further studies.

Preparation of insecticidal crystal protein and SDS-PAGE analysis

The spore–crystal mixture was isolated from the Bt isolates and the reference strain Btk HD-1, as described by Lenin *et al*. [17]. A single colony of each isolate and the reference strain was inoculated into 5 mL T_3 broth and incubated at 30°C for 60 h with 200 rpm shaking conditions. Sporulation was monitored under a light microscope, when more than 90% of the cells were lysed, the sporulated broth culture was shifted to 4°C, at least half-a-hour before harvesting. The T3 broth containing spore–crystal mixture was centrifuged for 10 min at 10,000 rpm at 4°C. The pellet was washed once with 5 ml of ice-cold Tris–EDTA buffer [Tris-10 mM, EDTA-1 mM, pH 8.0 with 1 mM phenyl methane sulphonyl fluoride (PMSF)], and 5 mL of ice-cold 0.5 M NaCl followed by 2 washes with 5 mL of Tris–EDTA buffer with 0.5 mM PMSF by centrifuging for 10 min at 10,000 rpm. Finally, the spore–crystal pellet was suspended in 100 µL of sterile distilled water containing 1 mM PMSF and stored in -20ºC. An aliquot of 10 µL spore–crystal mixtures of each Bt isolate and the reference strain was analysed by SDS-PAGE [18].

RESULTS AND DISCUSSION

Isolation of Bt

Of all the samples collected across different regions and diverse habitats of Andhra Pradesh, Gujarat, Haryana, Himachal Pradesh, Maharashtra, Rajasthan, Tamil Nadu, and Uttar Pradesh in India (Fig 1), 150 (37.5%) were found to contain Bt isolates. The occurrence of Bt was the highest in samples from Rajasthan (20%) (Fig 2). All the isolates formed creamy white, rough and entire undulate colonies. All the isolates were found to be rod shaped, gram-positive, spore forming. The colony characteristics of the isolates showed a slight variation from each other which is in line with the findings of Chaterjee *et al*. [19]. The reference strain also formed creamy white colonies, but with undulated margin.

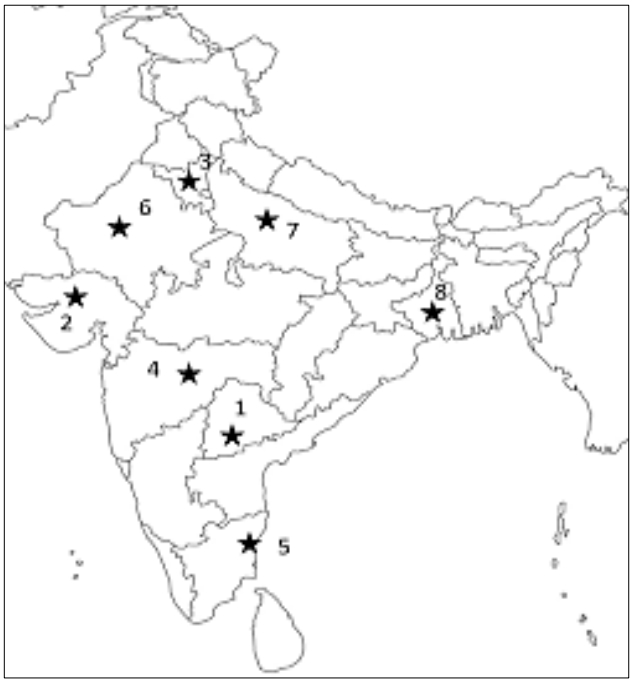

Fig 1 India map showing the states from where samples were collected

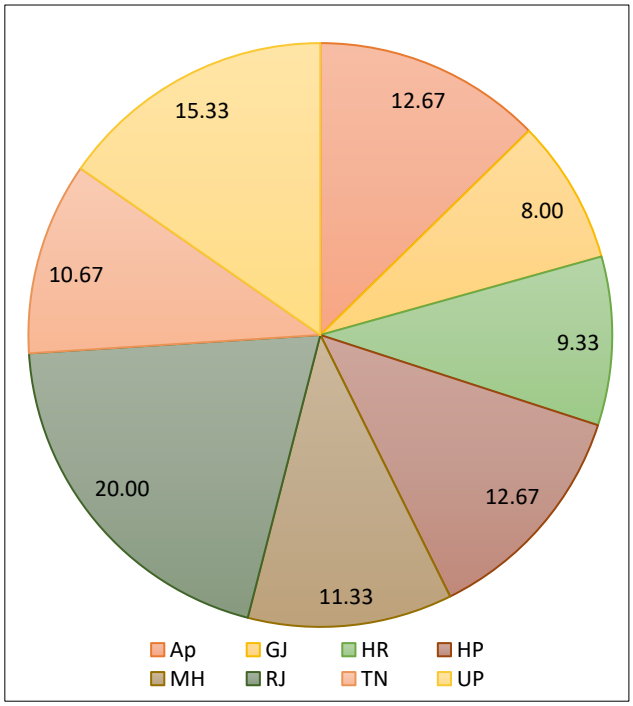

Fig 2 Pie chart showing the percentage of Bt isolated from each state

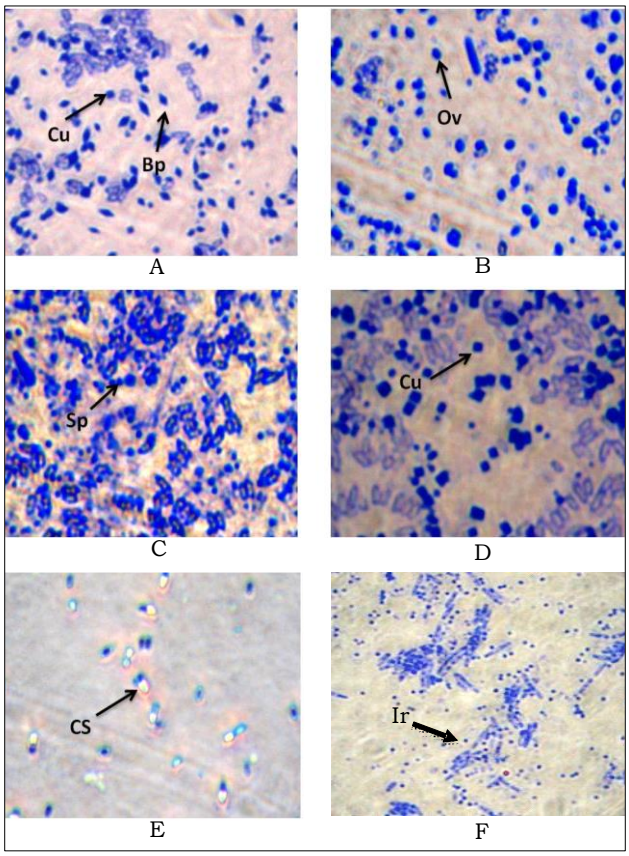

Fig 3 Light microscope photographs of ICPs formed by the Bt isolates

A: *Bipyramidal and cuboidal;* B: *Oval;* C: *Spherical;* D: *Cuboidal,* E: *Capped spores* and F: *Irregular*

Identification of Bt

Vegetative cell, spore and insecticidal crystal protein morphology

The presence of a parasporal inclusion body is a diagnostic feature to discriminate *Bt* form its close relatives in the *Bacillus cereus* group [20]. Based on the presence of parasporal inclusion, 150 out of 10,269 bacilli like *isolates* were classified as *Bt*. The morphological features of the vegetative cells were characteristic of Bt. All the isolates were rod shaped and gram positive. The spore positions of all isolates were sub-terminal. Major differences in the morphology and size of crystalline inclusions of native Bt isolates were observed by phase contrast microscopy. The results showed six different crystal morphologies like Bipyramidal & cuboidal (Bp & Cu), cuboidal (Cu), spherical (Sp), oval (Ov), irregular (Ir) and capped spores (Cs) (Fig 3), and the spore position in the isolates. In most isolates, parasporal inclusions were produced outside the endospore and were distinctly separated from it. But, the isolates BRI-33 from Haryana and BRI-112 from Tamil Nadu formed capped spores in which crystals were attached to the spores (Fig 3E). The Sp (73%) and Bp & Cu (17%) crystal morphologies were the most frequent type being present in the isolates from all regions (Table 1). Typically, 26 isolates were found to produce more than one type of crystal (Bipyramidal and cuboidal, Fig 3A). While Oval, Cuboidal, and irregular ICPs were formed by 4.6, 2.6, and 1.3 percent of the isolates respectively. The differences in the ICP morphology might be due to the genetic variation caused by the differences in the environmental conditions or the habitat effects [21].

Insecticidal crystal protein profiling

Crystal protein profiling of 150 Bt isolates and the reference strain Btk HD-1 was carried out by SDS-PAGE. Protein bands of various molecular weights representing different ICPs were observed in native Bt isolates (Table 1, Fig 6). The crystal proteins of the reference strain Btk HD-1 were resolved to 135 and 65 kDa sizes, while the molecular weight of proteins in Bt isolates were found to be in the range of 45–135 kDa (Fig 6). Of 150 Bt analyzed by SDS-PAGE, 26 isolates forming Bp & Cu crystals have exhibited bands of 130 and 60, while 109 Bt with Sp crystals, 7 with Ov crystals, 4 with Cu crystals, 2 with Cs crystals, and 2 with irregular crystals have exhibited bands of 135, 135 & 120, 45, 80 & 55, and 45 kDa, respectively.

Bt isolation in this study revealed their widespread occurrence, which is in conformity with the results of Chen *et al*. [22], and Chak *et al*. [23]. All the Bt isolates along with the reference strain Btk HD-1 were gram positive, rod shaped and endospore forming. In this study, 400 soil samples from 8 different states of India were used as source material for isolating indigenous Bt. Results revealed that 150 of the 400 samples were positive for Bt and yielded 150 isolates. Earlier studies reported varied frequencies for isolating Bt from soil samples ranging from 3 to 85% [24]. The samples were collected from both agricultural and non-agricultural soils. The highest percentage of Bt isolates is from Rajasthan (20%) followed by Uttar Pradesh (15%) could be due to the locations selected for soil sample collection in these two states. Most of the locations selected for collecting samples were hills regions and the barren lands which were not sprayed with the pesticides and undisturbed for a long period. The initial identification of Bt isolates was performed mainly on the presence of crystalline inclusions. In this study, 150 of the 10,269 stained bacterial colonies observed through light microscope showed crystalline inclusions, and were identified as Bt. Different crystal morphologies like bipyramidal and cuboidal, cuboidal, spherical, oval, capped spores, and irregular were observed. Shishir *et al*. [25] also identified Bt isolates based on the presence of parasporal crystal proteins and crystal protein profile and observed five different types of parasporal crystal proteins such as spherical, bipyramidal, irregular, pointed, cuboidal and irregular.

Table 1 Bt isolates their ICP morphology and protein profiling

Isolate	Location	Crystal Shape	ICP Mol. Wt. (kDa)	Isolate	Location	Crystal Shape	ICP Mol. Wt. (kDa)
BRI-1	AP	Sp	130	BRI-76	MH	Sp	130
BRI-2	AP	Sp	130	BRI-77	MH	Ov	120 &130
BRI-3	AP	Sp	130	BRI-78	MH	Sp	130
BRI-4	AP	Sp	130	BRI-79	MH	Sp	130
BRI-5	AP	Bp & Cu	130 & 60	BRI-80	MH	Sp	130
BRI-6	AP	Bp & Cu	130 & 60	BRI-81	MH	Sp	130
BRI-7	AP	Sp	130	BRI-82	RJ	Sp	130
BRI-8	AP	Sp	130	BRI-83	RJ	Cu	45
BRI-9	AP	Sp	130	BRI-84	RJ	Sp	130
BRI-10	AP	Sp	130	BRI-85	RJ	Sp	130
BRI-11	AP	Bp & Cu	130 & 60	BRI-86	RJ	Bp & Cu	130 & 60
BRI-12	AP	Sp	130	BRI-87	RJ	Sp	130
BRI-13	AP	Sp	130	BRI-88	RJ	Sp	130
BRI-14	AP	Sp	130	BRI-89	RJ	Sp	130
BRI-15	AP	Bp & Cu	130 & 60	BRI-90	RJ	Sp	130
BRI-16	AP	Bp & Cu	130 & 60	BRI-91	RJ	Sp	130
BRI-17	AP	Sp	130	BRI-92	RJ	Sp	130
BRI-18	AP	Sp	130	BRI-93	RJ	Ov	120 &130
BRI-19	AP	Sp	130	BRI-94	RJ	Bp & Cu	130 & 60
BRI-20	GJ	Sp	130	BRI-95	RJ	Sp	130
BRI-21	GJ	Sp	130	BRI-96	RJ	Sp	130
BRI-22	GJ	Sp	130	BRI-97	RJ	Sp	130
BRI-23	GJ	Sp	130	BRI-98	RJ	Sp	130
BRI-24	GJ	Sp	130	BRI-99	RJ	Sp	130
BRI-25	GJ	Sp	130	BRI-100	RJ	Sp	130
BRI-26	GJ	Sp	130	BRI-101	RJ	Sp	130
BRI-27	GJ	Sp	130	BRI-102	RJ	Sp	130
BRI-28	GJ	Sp	130	BRI-103	RJ	Sp	130
BRI-29	GJ	Sp	130	BRI-104	RJ	Sp	130
BRI-30	GJ	Sp	130	BRI-105	RJ	Sp	130
BRI-31	GJ	Bp & Cu	130 & 60	BRI-106	RJ	Sp	130
BRI-32	HR	Sp	130	BRI-107	RJ	Ov	120 &130
BRI-33	HR	Cs	80 & 55	BRI-108	RJ	Ov	120 &130
BRI-34	HR	Bp & Cu	130 & 60	BRI-109	RJ	Sp	130
BRI-35	HR	Sp	130	BRI-110	RJ	Sp	130
BRI-36	HR	Sp	130	BRI-111	RJ	Bp & Cu	130 & 60
BRI-37	HR	Sp	130	BRI-112	TN	Cs	80 & 55
BRI-38	HR	Cu	45	BRI-113	TN	Ov	120 &130
BRI-39	HR	Sp	130	BRI-114	TN	Sp	130
BRI-40	HR	Bp & Cu	130 & 60	BRI-115	TN	Sp	130
BRI-41	HR	Sp	130	BRI-116	TN	Sp	130
BRI-42	HR	Bp & Cu	130 & 60	BRI-117	TN	Ir	45
BRI-43	HR	Sp	130	BRI-118	TN	Bp & Cu	130 & 60
BRI-44	HR	Sp	130	BRI-119	TN	Sp	130
BRI-45	HR	Sp	130	BRI-120	TN	Cu	45
BRI-46	HP	Bp & Cu	130 & 60	BRI-121	TN	Bp & Cu	130 & 60
BRI-47	HP	Sp	130	BRI-122	TN	Sp	130
BRI-48	HP	Ov	80 & 45	BRI-123	TN	Bp & Cu	130 & 60
BRI-49	HP	Sp	130	BRI-124	TN	Bp & Cu	130 & 60
BRI-50	HP	Sp	130	BRI-125	TN	Sp	130
BRI-51	HP	Bp & Cu	130 & 60	BRI-126	TN	Sp	130
BRI-52	HP	Bp & Cu	130 & 60	BRI-127	TN	Sp	130
BRI-53	HP	Ir	45	BRI-128	UP	Sp	130
BRI-54	HP	Sp	130	BRI-129	UP	Sp	130
BRI-55	HP	Sp	130	BRI-130	UP	Sp	130
BRI-56	HP	Sp	130	BRI-131	UP	Sp	130
BRI-57	HP	Sp	130	BRI-132	UP	Ov	120 &130
BRI-58	HP	Sp	130	BRI-133	UP	Bp & Cu	130 & 60
BRI-59	HP	Sp	130	BRI-134	UP	Sp	130
BRI-60	HP	Sp	130	BRI-135	UP	Sp	130
BRI-61	HP	Sp	130	BRI-136	UP	Bp & Cu	130 & 60
BRI-62	HP	Sp	130	BRI-137	UP	Bp & Cu	130 & 60
BRI-63	HP	Sp	130	BRI-138	UP	Sp	130

BRI-64	HP	Sp	130	BRI-139	UP	Sp	130
BRI-65	MH	Sp	130	BRI-140	UP	Sp	130
BRI-66	MH	Sp	130	BRI-141	UP	Sp	130
BRI-67	MH	Sp	130	BRI-142	UP	Sp	130
BRI-68	MH	Sp	130	BRI-143	UP	Sp	130
BRI-69	MH	Bp & Cu	130 & 60	BRI-144	UP	Cu	45
BRI-70	MH	Sp	130	BRI-145	UP	Sp	130
BRI-71	MH	Sp	130	BRI-146	UP	Sp	130
BRI-72	MH	Sp	130	BRI-147	UP	Bp & Cu	130 & 60
BRI-73	MH	Sp	130	BRI-148	UP	Bp & Cu	130 & 60
BRI-74	MH	Bp & Cu	130 & 60	BRI-149	UP	Sp	130
BRI-75	MH	Bp & Cu	130 & 60	BRI-150	UP	Sp	130

AP- Andhra Pradesh, GJ- Gujarat, HR- Haryana, HP- Himachal Pradesh, MH- Maharashtra, RJ- Rajasthan, TN- Tamil Nadu, UP- Uttar Pradesh
*Bp & Cu- Bipyramidal and cuboidal, Cs- Capped Spores, Cu- Cuboidal, Ir- Irregular, Ov- Oval, and Sp- Spherical

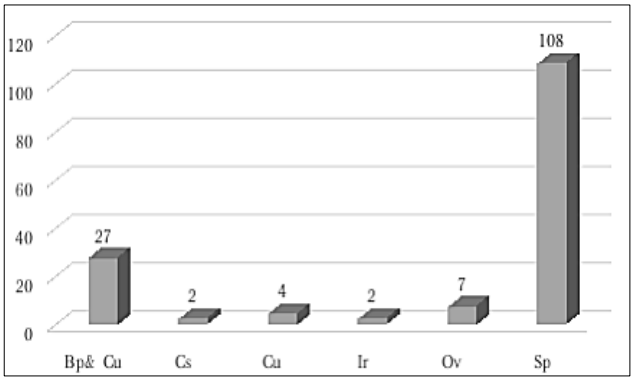

Fig 4 Graphical representation of Bt isolates categorized based on the ICPs formed by the respective isolates

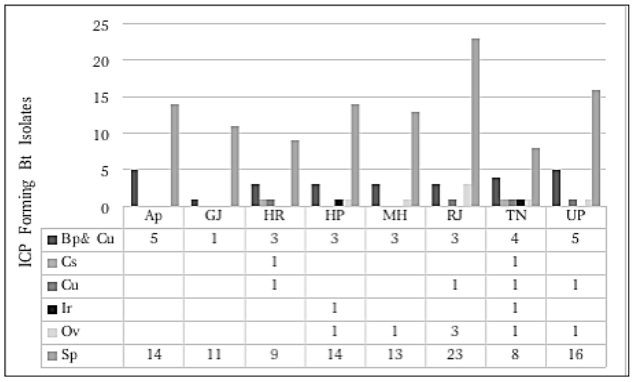

Fig 5 Graph showing the distribution of Bt isolates state wise. AP: Andhra Pradesh, GJ: Gujarat, HR: Haryana, HP: Himachal Pradesh, MH: Maharashtra, TJ: Rajasthan, TN: Tamil Nadu, and UP: Uttar Pradesh. Bp & Cu- Bipyramidal and cuboidal, Cs- capped spores, Cu- cuboidal, Ir- Irregular, Ov- Oval, and Sp- Spherical

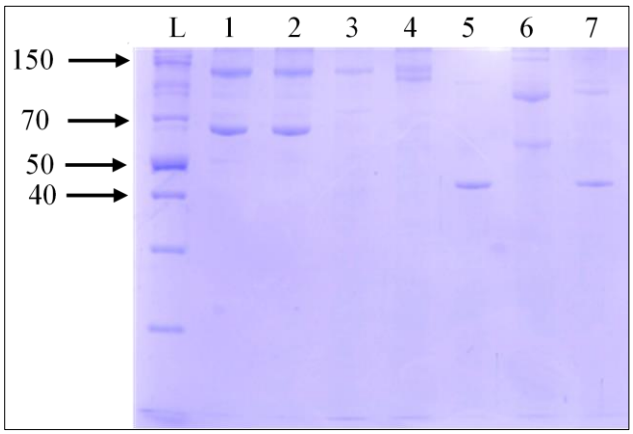

Fig 6 SDS-PAGE profiling of ICPs isolated from Bt. L: Protein molecular weight marker (10-200 kDa); Lanes 1: Reference strain, Btk HD-1; 2: Bp & Cu; 3: Sp: 4: Ov; 5: Cu; 6: Cs; 7: Ir.

About 72.66% of the 150 Bt formed spherical crystals, followed by bipyramidal crystals in 17.33%. These findings differed from the earlier reports [26-27], in which the strains with bipyramidal crystals (46%) and cuboidal (27%) were predominant in the respective studies. In addition to these shapes, oval (4.66%), cuboidal (2.66%), irregular (1.33%) and capped spores (1.33%) were observed in this study. Rampersad and Ammons [28] also reported the capped spores in their study. The varying shapes of crystalline inclusions suggest the presence of diversity in the Bt isolates obtained from different regions in India. Protein profiles by SDS-PAGE from crystal inclusions have been used routinely for the differentiation and characterization of Bt to determine the main entomopathogenic factors [29]. Protein analyses of the crystal-spore preparations showed delta-endotoxin with diverse electrophoretic patterns with molecular weights in the range of 60–140 kDa. Earlier studies reported that the proteins with a molecular weight of 132 kDa are encoded by the c*ry1* and c*ry4* genes [30]. Proteins with bipyramidal inclusions encoded by c*ry1* genes are active against lepidopteran insects [30] whereas those with spherical inclusions encoded by c*ry4* genes have great pathogenic variability with activity against dipterans, nematodes, mites, and lepidoptera [31]. The proteins in the range of 60–80 kDa with cuboidal inclusions are encoded by *cry2*, *cry3* genes [30] and *cry10* or *cry11* genes [32] and are active against lepidopteran, dipteran and Coleopteran insects [33]. In this study, 17.33% of the 150 isolates have formed crystalline inclusions with a molecular weight of 130 and 60 kDa proteins, suggesting the presence of genes associated with the *cry1* and *cry2* families. Other isolates showed 60–80 kDa proteins, indicating other novel *cry* genes. These results revealed wider genetic diversity in Bt isolates from various regions of India. Further studies on the molecular diversity of Bt isolates, *cry* gene screening, cloning and characterization of *cry* genes from these new isolates of Bt will be useful in the area of integrated pest management for sustainable agriculture.

CONCLUSION

Results revealed that the screening methodology used in this study might deliver an abundant and potentially more useful collection of Bt isolates compared with those obtained using commonly reported methodologies. Crystal protein profiling suggests that the Bt isolates isolated in this study may harbour novel *cry* genes encoding proteins active against Lepidoptera and other classes of insects.

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