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Screening of indigenous *Bacillus thuringiensis* for dipteran active *cry* gene profiles and potential toxicity against melon fruit fly, *Zeugodacus cucurbitae* (Coquillett)

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Abstract

Background Melon fruit fly, *Zeugodacus cucurbitae* Coquillett (Diptera: Tephritidae), is a devastating polyphagous pest attacking large number of fruits and vegetables causing huge economic yield losses across the world. Management of this notorious pest is very challenging as the larvae feed inside the fruit. Hence, the present research study aimed to screen the indigenous *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) strains causing toxicity to larvae and to identify the insecticidal toxicity-related genes present in respective strains. In the present study, 50 indigenous *B. thuringiensis* (*Bt*) strains along with one reference strain were screened against second-instar larvae *Z. cucurbitae*. All the strains were analyzed for presence of 21 dipteran active *cry* genes.

Results Mortality in *Z. cucurbitae* larvae due to *Bt* strains ranged from 16 to 92%. PCR results revealed that each strain tested positive for a minimum of three *cry* genes to maximum of nine *cry* genes. Among the *cry* genes, *cry1A*, *cry2A*, *cry1C*, *cry19*, *cry11* and *cry70* were detected in high frequency of 100, 88, 84, 74, 58 and 54%, respectively. Bioassay studies revealed that ten out of fifty strains displayed more than 50% mortality. Hence, these ten strains, along with the reference strain, were further tested for mortality for the calculation of the median lethal concentration (LC_{50}). The LC_{50} values ranged between 38.48 and 105.18 µg/ml. The lowest LC_{50} found for the strain NBAIR Bt107 was 38.48 µg/ml and was on par with the reference strain (Bti 4Q1) (31.3 µg/ml).

Conclusion Indigenous *Bt* strains displayed a toxicity against the larvae of *Z. cucurbitae*. The probable dipteran active *cry* genes responsible for toxicity were interpreted. Thus, the Cry toxins from *Bt* can play a very important role in the management of *Z. cucurbitae*.

Keywords *Zeugodacus cucurbitae*, *Bacillus thuringiensis*, Bioassay, Crystal proteins, Median lethal concentration

Background

Zeugodacus cucurbitae Coquillett (Diptera: Tephritidae), commonly known as the melon fruit fly, is a serious pest belonging to the family Tephritidae of the order Diptera. This highly notorious species is capable of infesting over 100 different varieties of vegetables and fruits, making it a major concern for crop yield and food security (Dhillon et al. 2005). The level of damage caused by this pest can range from 30 to 100% (Subedi et al. 2021). Melon fruit fly not only causes direct damage by reducing the yield

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and marketability of fruits and vegetables but also presents substantial challenges to quarantine security. Thus, it poses a threat to the international trade of fruits and vegetables on a global scale (Vargas et al. 2015).

The management practices predominantly depend on the use of synthetic pesticides mainly as cover sprays due to their rapid knockdown effect (De Bon et al. 2014). These cover sprays not only trigger ecological repercussions in fruit fly populations, such as the development of insecticide resistance, but also lead to unintended harm to non-target beneficial arthropods and can induce phytotoxic effects on plants (Ankitha et al. 2023). There is a need for alternate management approaches that efficiently control this pest while keeping non-target organisms safe. So, the use of microbial control agents, in the form of biopesticides, could be a promising alternative (Ruqiya et al. 2023). In this context, the use of *Bt* provides a promising opportunity for the management of *Z. cucurbitae*. With its proven efficacy against a diversity of insect groups and considering its relatively underexplored status in testing toxicity against *Z. cucurbitae*, it provides a large scope for the identification of indigenous *Bt* strains effective against melon fruit fly.

Bacillus thuringiensis is a Gram-positive, spore-forming aerobic saprophytic bacterium of ubiquitous nature. Because of its high specificity, safety, quick degradation from soil, and consistent pest control, it is the most widely accepted commercial biopesticide across the globe (Manjunatha et al. 2023). The insecticidal property of *Bt* is due to the production of toxins during different phases of its life cycle. *Bt* strains can synthesize crystal and cytolytic toxins called Cry and Cyt toxins widely called as δ-endotoxins. Upon ingestion by insect pests, the activation of these toxins takes place within the midgut, facilitated by midgut proteases. Once activated, the toxins bind to specific receptors on the midgut epithelial cells, ultimately disrupting cell function and causing cell death (Bravo et al. 2007). The efficacy of these toxins extends beyond insect pests of the order Lepidoptera to Diptera, including those belonging to the Tephritidae family of agricultural importance. A few of these reports include the toxicity of *Bt* against the Mexican fruit fly, *Anastrepha ludens* Loew (Martinez et al. 1997), the peach fruit fly, *Bactrocera zonata* Saunders (Nisar et al. 2020) and the olive fruit fly *B. oleae* Rossi (Ilias et al. 2013). However, there are only limited studies on the evaluation of the toxicity of indigenous *Bt* strains against the melon fruit fly. Moreover, this is the first report from India to evaluate the toxicity of indigenous *Bt* against *Z. cucurbitae*.

Each strain of *Bt* may possess a diverse array of *cry* genes. Researchers have identified nearly 700 *cry* genes from 75 families to date (Crickmore et al. 2021). Each toxin exhibits high specificity toward one or a group of closely related insects. Furthermore, the presence of specific combinations of genes in each strain strongly influences its toxic activity against insect pests. Therefore, the identification of *cry* gene profiles within a strain offers a significant opportunity to understand and interpret its insecticidal activity. Hence, in the present study, the detection of dipteran active toxic genes using PCR was done in all indigenous strains of the study. The strains with different combinations of *cry* genes were screened to determine their potential toxicity against *Z. cucurbitae*.

Methods

Source of *Bacillus* thuringiensis strains

A total of fifty *Bt* is strains, along with the dipteran active strain *Bt* var. *israelensis*, were collected from the Insect Bacteriology Laboratory, Division of Genomic Resources, ICAR-National Bureau of Agricultural Insect Resources, Bengaluru, Karnataka, India. All the strains were revived on T3 broth then incubated for four days and plated on LB media and preserved in glycerol stock for further use.

Toxicity analysis of *Bacillus* thuringiensis against Zeugodacus cucurbitae

Insect rearing

Fruit fly culture was raised from the infested *Cucumis sativus* Linnaeus (Cucurbitales: Cucurbitaceae) fruits that were collected from cucumber fields in the Chikkaballapur district of Karnataka. Infested melon fruits were brought and kept in a glass jar ($8'' \times 6''$) under controlled conditions of $25 \pm 2°$ C, $70 \pm 5\%$ RH. The glass jar was filled with sterilized sand for pupation, allowing full-grown fourth-instar larvae to emerge from infested fruits and pupate in soil. Upon adult emergence, ten pairs of males and females were collected and released in a cage ($30 \times 30 \times 30$ cm). Adults were provided with a mixture of yeast hydrolysate and sugar (1:3) as a source of food and a water-soaked cotton swab as a source of water. This was used as a mother culture. Equal pairs of males and females were transferred from the mother culture to ovipositional cages provided with a piece of cucumber for oviposition. The eggs were collected and seeded over the artificial diet as recommended by Liu et al. (2020).

Preparation of spore-crystal suspension

Cells from each *Bt* isolate were inoculated into 100 ml of T3 broth and incubated at 30° C for 3 days. On the fourth day, a microscopic examination was conducted to identify the presence of crystals. From the isolates that tested positive for crystals, the spore-crystal mixture was harvested by centrifuging the inoculated broth separately at 15,000 rpm and 4° C for 10 min.

The resultant pellet was washed three times with sterile distilled water before being re-suspended in sterile distilled water and used for bioassays as described by Ammouneh et al. (2011).

Screening of Bacillus thuringiensis isolates against Zeugodacus cucurbitae

Screening of *Bt* isolates against second-instar larvae of *Z. cucurbitae* was performed using the diet incorporation with a spore-crystal suspension. The mortality caused by the fifty *Bt* isolates was compared with reference strain, *Bt* var. *israelensis* (strain 4Q1). All isolates, along with the reference strain, were screened against second-instar larvae at a single concentration (250 µg/ml). Exactly, 100 µl of spore-crystal homogenate incorporated into 10 g of artificial diet and transferred to small plastic containers (3 × 3 cm), with each container serving as a replicate. Five larvae for each replicate, 25 larvae for each concentration and three replications were used for each treatment. The bioassay was carried out under controlled conditions of 25 ± 2° C and 70 ± 5% relative humidity. Mortality percentage was calculated at 24-h intervals till the fifth day of the experiment. Concentration- response bioassay using different concentrations (250, 125, 62.5, 31.25 or 15.62 µg/ml) was carried out in a similar manner to the single concentration assay. Based on the single concentration assay, the strains caused more than 50% mortality in *Z. cucurbitae* larvae were chosen for this bioassay. Five larvae were used for each replicate and 25 larvae for each concentration (250, 125, 62.5, 31.25 or 15.62 µg/ml). There were five replicates for each treatment.

PCR amplification of dipteran active cry genes

Total DNA was extracted from all *Bt* isolates using DNeasy Blood and Tissue Kit, following the manufacturer's protocol (Qiagen, Hilden, Germany). The DNA from each strain individually used as template for amplification of the dipteran active *cry* gene in a PCR mixture of 25 µl containing 12.5 µl of 2 × EmeraldAmp PCR master mix (Emerald Amp GT PCR master mix, TaKaRa, Japan), 1 µl of forward primer, 1 µL of reverse primer, 5.5 µl of molecular biology grade water and 5 µl of template DNA (Valtierra-de-Luis et al. 2020; Nanditha et al. 2024). The reaction was set up at 94° C for 4 min as pre-denaturation, followed by 35 cycles of denaturation at 94° C for 1 min, annealing for 1 min at a temperature specific to each primer, extension at 72° C for 1 min and a final extension for 5 min at 72° C; annealing temperatures for each primer are given in (Table 1). The amplified products were visualized in 1.5% agarose gel and documented under the gel documentation system (DNR, MiniLumi, Israel).

Statistical analysis

The experiment was laid out as a completely randomized design. All the assays were performed three times with five replicates of each treatment. The concentration–response relationships for mortality of larvae were analyzed by subjecting data to probit analysis (PROC PROBIT) using SAS software (version 9.3, 2011; SAS Institute, Cary, NC, USA) and used as the basis for the calculation of LC_{50} and LC_{90} values and their corresponding 95% fiducial limit (95% FL).

Results

Bioassay of *Bacillus* thuringiensis strains against Zeugodacus cucurbitae

The evaluation of *Bt* strains for potential toxicity against second-instar larvae of *Z. cucurbitae* indicated that the mortality percentage ranged from 16 to 92% in comparison to the reference strain, which showed 95% mortality. Mortality of the larvae due to *Bt* confirmed by re-isolating *Bt* from dead larvae (Fig. 1). Ten out of fifty strains (NBAIR Bt101, NBAIR Bt103, NBAIR Bt104, NBAIR Bt107, NBAIR Bt112, NBAIR Bt113, NBAIR Bt114, NBAIR Bt119, NBAIR Bt120 and NBAIR Bt142) caused more than 50% mortality (Table 2). LC_{50} and LC_{90} values of all the ten strains ranged 38.48–105.18 (µg/ml) and 146–150 (µg/ml), respectively, at 120 h (five days) after treatment. The highest percent mortality and lowest LC_{50} value were observed for NBAIR Bt107, and it was on par with the mortality caused by the reference strain (Bti-4Q1) (Table 3).

Frequency and distribution of dipteran active *cry* genes

To dwell into the *cry* gene profiles of each strain and correlate them with differences in mortality percentage, 50 strains for amplification of twenty-one dipteran active *cry* genes were tested (Table 2). The strains that displayed desired-size amplification products on agarose gel were regarded as positive for the corresponding genes (Additional file 1: Fig. S1). The frequency of *cry* genes present in all strains was identified, summarized and depicted in Fig. 2. The results showed each strain harbored at least one or other dipteran active *cry* genes, but none of the strains tested positive for *cry4A*, *cry4B*, *cry29* and *cry44* genes.

All the strains (50/50) tested positive for *cry1A*, followed by the *cry2A* gene in 88% (44/50) and *cry1C* gene in 84% (42/50) of strains that were found. Among the different *cry1* subfamilies, *cry1B* was the least abundant, as

Table 1 Details of PCR primers used in the study for dipteran active *cry* gene profiles of indigenous *Bacillus thuringiensis* strains

Sl. No	*cry* gene		Primer sequence	Amplicon size (bp)	References
1	*cry1A*	*cry1A*F	ATTCGCTAGGAACCAAGC	398	Thammasittirong and Attathom (2008)
		*cry1A*R	AATCCGGTCCCCATACAC		
2	*cry1B*	*cry1B*F	CAGAAACAACAGAACGACC	921	Thammasittirong and Attathom (2008)
		*cry1B*R	CACTTCCCCACCATCCAT		
3	*cry1C*	*cry1C*F	TAATCCACAGTTACAGTC	432	Thammasittirong and Attathom (2008)
		*cry1C*R	TATTATCCTCAGGCGGTAA		
4	*cry2A*	*cry2A*F	CGATATGTTAGAATTTAGAAC	1170	Porcar and Perez (2003)
		*cry2A*R	-TACCGTTTATAGTAACTCG		
5	*cry4A*	*cry4A*F	TCAAAGATCATTTCAAAATTACATG	459	Bukhari et al. (2022)
		*cry4A*R	CGGCTTGATCTATGTCATAATCTGT		
6	*cry4B*	*cry4B*F	GGGTATGGCACTCAACCCCACTT	1925	Ben Dov et al. (1997)
		*cry4B*R	GCGTGACATACCCATTTCCAGGTCC		
7	*cry10A*	*cry10A*F	ATAAATTCAAGTGCCAAGTA	490	Porcar and Perez (2003)
		*cry10A*R	CCGAACCTACTATTGCGCCA		
8	*cry11A*	*cry11A*F	CCGAACCTACTATTGCGCCA	445	Ben Dov et al. (1997)
		*cry11A*R	CTCCCTGCTAGGATTCCGTC		
9	*cry16A*	*cry16A*F	TCAAAAGGTGTGGCAAG	1415	Barloy et al. (1998)
		*cry16A*R	ATAAGCCCAATATCATG		
10	*cry19A*	*cry19A*F	AGGGGAGTCCAGGTTATGAG	755	Ejiofor and Johnson (2002)
		*cry19A*R	ATTTCCCTAGTTAGTTCGGTTTTT		
11	*cry20A*	*cry20A*F	CAATCCCTGGCTTCACTCGT	490	Ejiofor and Johnson (2002)
		*cry20A*R	CCGCGGGCATTAGGATT		
12	*cry21*	*cry21*F	ATACAGGGATAGGATTTCAAG	453	Ejiofor and Johnson (2002)
		*cry21*R	ATCCCCATTTTCTATAAGTGTCT		
13	*cry24C*	*cry24C*F	AGGGGGCGATGGATACGAC	495	Ejiofor and Johnson (2002)
		*cry24C*R	GGCCCTGCTACAACCGAAACTA		
14	*cry25*	*cry25*F	CGTTTTCCGCATTATCATTAGG	455	Ejiofor and Johnson (2002)
		*cry25*R	ACGCCCCGGCTGTCTTA		
15	*cry27A*	*cry27A*F	GTGGCATATAGACTAAGGGAGGAA	387	Ejiofor and Johnson (2002)
		*cry27A*R	TTGCAGGCCATATAAGAGGTGTT		
16	*cry29*	*cry29*F	TCAGCTCCAATAACTGGTG	451	Ibarra et al. (2003)
		*cry29*R	GCATGTCATCCCCTTGTCTA		
17	*cry30*	*cry30*F	AACTCACACATCCTCCATCG	265	Ibarra et al. (2003)
		*cry30*R	ATCGGAAGGCAATCATTCG		
18	*cry32B*	*cry32B*F	TGGTCGGGAGAGAATGGATGGA	677	Ibarra et al. (2003)
		*cry32B*R	TGGTCGGGAGAGAATGGATGGA		
19	*cry39A*	*cry39A*F	AAGCTGCGAATCTGCATTTACTTTT	319	Ibarra et al. (2003)
		*cry39A*R	CTCATAATTTTCCGTCCATAAAT		
20	*cry44A*	*cry44A*F	ACAAATT ATAAAGATTGGCT	1800	Ito et al. (2006)
		*cry44A*R	GAGTAATTGGCAGAAATTC-3		
21	*cry70*	*cry70*F	TCCCACCAGTCCCACCTATT	992	This study
		*cry70*R	GGGCGATGATCTGTTAGGCT		

only 12 strains harbored this gene. Similarly, the genes *cry25* and *cry27* were the least abundant among all the *cry* genes present, as only 0.0 2% (1/50) of the strains tested positive for those genes. Only three and ten strains among 50 tested positive for *cry21* and *cry39* genes, respectively. Also, twenty-seven and thirty-seven strains among 50 strains tested positive for *cry70* and *cry27* genes, respectively. Similarly, 28, 58, 74, 24, 16, 14 and 22% of strains tested positive for *cry10*, *cry11*, *cry19*, *cry24*, *cry25*, *cry30* and *cry32* in the same order, respectively.

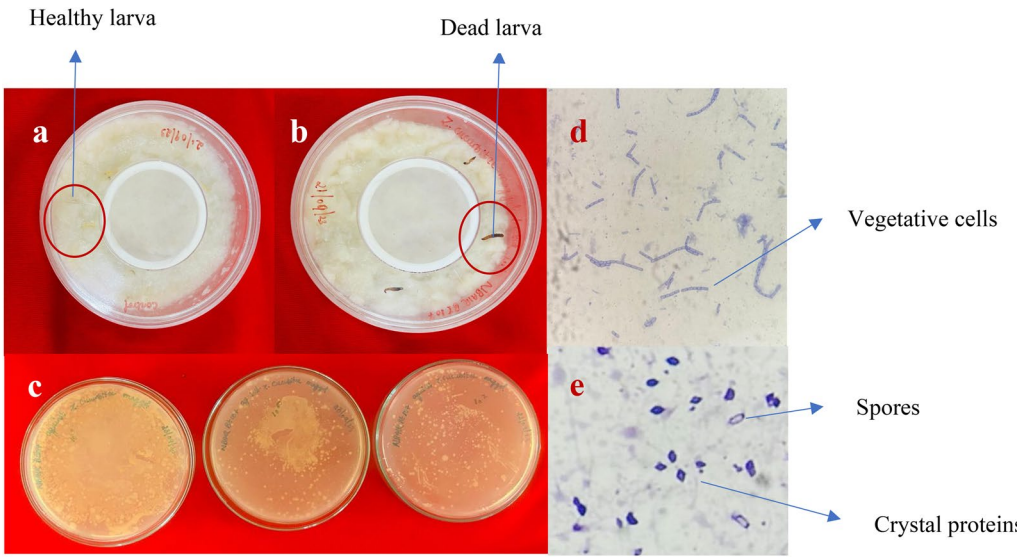

Fig. 1 Bioassay and re-isolation of *Bacillus thuringiensis* from cadaver of *Zeugodacus cucurbitae* to prove Koch's postulates **a** control: healthy larva not showing any discoloration of body; **b** *B. thuringiensis*-treated larva displaying complete discoloration of body; **c** Petri plates showing the colonies of *B. thuringiensis* reisolated from cadaver; **d** vegetative cells of *B. thuringiensis*; **e** spores and crystal proteins of *B. thuringiensis* re-isolated from dead larva under microscope

Diversity of dipteran active *cry* gene combinations

The study on *cry* gene combinations in *cry* gene-positive strains revealed that every strain contained a minimum of three *cry* genes, with certain strains harboring a maximum of nine *cry* genes. Three out of 50 strains (6%) tested positive for nine *cry* genes, but just two percent (1/50) of strains only had three *cry* genes. A total of 16% (8/50), 12% (6/50), 44% (22/50) and 10% (4/50) of strains were positive for eight, seven, six, five and four *cry* genes, respectively, in different combinations. However, *cry* gene profiles of each strain were found to be distinct. So, for ease of comparison of *cry* gene combinations with possible toxicity, the strains were grouped, based on the number of *cry* genes (Fig. 3). All the fifty strains were classified into seven groups based on the number of *cry* genes present in each strain (Table 2).

Among the four strains that had the highest number of *cry* genes, 100% of them (4/4) harbored *cry1A*, *cry1C*, *cry2A*, *cry11* and *cry70*, but all strains were found to be negative for gene *cry30*. But *cry10* tested positive for only two strains (NBAIR Bt101 and NBAIR Bt104) that showed high toxicity. Three strains tested positive for the *cry19* gene, only two strains tested positive for the *cry27* gene, and another two strains tested positive for the gene *cry25*. The genes *cry16* and *cry27* combination was found only in NBAIR Bt111; similarly, *cry32* and *cry39* combination was found only in NBAIR Bt121.

In addition to the commonly observed genes *cry1A*, *cry1C* and *cry2A*, a few other genes, such as *cry17* and *cry19*, were also prevalent among the thirteen strains that tested positive for either eight or seven *cry* genes. There were only two strains that carried either the *cry21* or *cry25* genes. The presence of *cry24* was more abundant, and *cry25* was the least abundant among the strains that tested positive for eight *cry* genes compared to those with seven *cry* genes. Ten strains showed positive results for *cry11*, and eleven strains are positive for *cry19*. Only a single strain harbored both *cry30* and *cry32*.

The highest number of strains (23/50) tested positive for six dipteran active *cry* genes in different combinations. Among these strains, the *cry* gene profiles in combination with *cry1A*, *cry1C*, *cry2A*, *cry11* and *cry19* are found to be more abundant. The only strain that was positive for *cry20* was also found positive for *cry24*, *cry70*, *cry10*, *cry2A* and *cry1A*. There was a huge correlation between *cry11* and *cry19*, as most of the strains that are positive for *cry11* were also found positive for *cry 19*. Within the strains that were positive for either three, four or five *cry* genes, association of *cry 19* with *cry1* was more frequent, and 60% of strains (6/10) had *cry* gene profiles of *cry1A*, *cry1B* and *cry 19*.

Comparison of cry gene profiles of *Bacillus* thuringiensis strains with toxicity against Zeugodacus cucurbitae larvae

Based on the PCR amplification of the dipteran active *cry* genes in all fifty strains, a diversity of *cry* gene combinations was identified. The *cry* gene combinations in a strain were compared to the mortality of second-instar

Table 2 Bracketing bioassay of *Bacillus thuringiensis* strains against second-instar larvae of *Zeugodacus cucurbitae* and their *cry* gene profiles

Sl. No	Strain name	Mortality (%)
Group I (Strains having nine *cry* genes)		
1	NBAIR Bt101	88
2	NBAIR Bt104	80
3	NBAIR Bt111	48
4	NBAIR Bt121	52
Group II (Strains having eight *cry* genes)		
5	NBAIR Bt105	44
6	NBAIR Bt113	76
7	NBAIR Bt114	84
8	NBAIR Bt136	36
9	NBAIR Bt141	40
10	NBAIR Bt142	88
11	NBAIR Bt144	36
12	NBAIR Bt146	48
Group III (Strains having seven *cry* genes)		
13	NBAIR Bt112	62
14	NBAIR Bt113	68
15	NBAIR Bt132	44
16	NBAIR Bt133	48
17	NBAIR Bt149	36
Group 1 V (Strains having six *cry* genes)		
18	NBAIR Bt102	40
19	NBAIR Bt103	56
20	NBAIR Bt107	92
21	NBAIR Bt108	40
22	NBAIR Bt109	32
23	NBAIR Bt110	40
24	NBAIR Bt115	24
25	NBAIR Bt116	46
26	NBAIR Bt117	48
27	NBAIR Bt118	44
28	NBAIR Bt119	76
29	NBAIR Bt120	84
30	NBAIR Bt124	24
31	NBAIR Bt125	32
32	NBAIR Bt134	36
33	NBAIR Bt137	24
34	NBAIR Bt138	16
35	NBAIR Bt139	24
36	NBAIR Bt140	12
37	NBAIR Bt145	28
38	NBAIR Bt147	40
39	NBAIR Bt148	32
40	NBAIR Bt149	36
Group V (Strains having five *cry* genes)		
41	NBAIR Bt127	44
42	NBAIR Bt128	32
43	NBAIR Bt129	36
44	NBAIR Bt135	48
45	NBAIR Bt143	40
Group VI (Strains having four *cry* genes)		
46	NBAIR Bt106	44
47	NBAIR Bt122	28
48	NBAIR Bt123	20
49	NBAIR Bt126	24
Group VII (Strains having three *cry* genes)		
50	NBAIR Bt131	16

larvae of *Z. cucurbitae* (Table 1). The number of *cry* genes present in the *Bt* strains that displayed more than 50% mortality tested positive for at least six active dipteran active *cry* genes to a maximum of nine *cry* genes. However, the greatest number of *cry* genes in a strain not necessarily causes greater mortality, but the all the *Bt* strains displayed more than 50% mortality showed a common combination of *cry1A*, *cry2A*, *cry10A* and *cry70*.

Discussion

Numerous effective *Bt* strains have been found worldwide, and their toxicity may vary depending on insect species and environmental factors. So, it is always essential to look for indigenous strains that are highly acquainted with the local environment from which they are isolated (Ma et al. 2023). India, being rich in a diversity of habitats, offers ample opportunities for the identification, characterization and exploration of native effective *Bt* strains. Several studies have documented the insecticidal toxicity of *Bt* against multiple insect orders, including Diptera (Fernández-Chapa et al. 2019).

In the present study, a total of fifty *Bt* strains were screened for dipteran active *cry* genes and toxicity against *Z. cucurbitae*. The strong insecticidal property of any *Bt* strain is highly correlated with the combined activity of different *cry* genes in a particular strain. Hence, the identification of *cry* gene content forms a basis for the prediction of possible toxicity against different species of insects. Based on PCR amplification analysis, dipteran active *cry* genes such as *cry1A*, *cry1B*, *cry1C*, *cry2A*, *cry10*, *cry11*, *cry16*, *cry19*, *cry20*, *cry21*, *cry24*, *cry25*, *cry27*, *cry30*, *cry32*, *cry39* and *cry70* were reported in native *Bt* strains at different frequencies. None of the isolates were tested positive for *cry4A*, *cry4B*, *cry29* or *cry44*. In the present study, among *cry1* subfamilies, *cry1A* was present in all strains, and only 24% (12/50) and 84% (42/50) *cry1A* gene-positive strains also harbored *cry1B* and *cry1C*, respectively. The *cry1A* and *cry2A* gene

Table 3 Bioassay of indigenous *Bacillus thuringiensis* strains showing more than 50% of mortality of *Zeugodacus cucurbitae* larvae

Sl. No	Strain name	LC_{50} (µg/mL)	95% Fiducial limit	Slope ± SE	χ2	P
1	NBAIR Bt101	41.35	26.16–60.31	2.36 ± 0.52	20.26	0.90
2	NBAIR Bt103	105.18	68.37–192.31	2.53 ± 0.56	20.40	0.17
3	NBAIR Bt104	61.38	41.30–91.50	2.65 ± 0.54	23.65	0.89
4	NBAIR Bt107	38.48	22.60–56.62	2.09 ± 0.52	15.92	0.82
5	NBAIR Bt112	58.54	40.77–81.22	3.09 ± 0.58	27.83	0.91
6	NBAIR Bt113	64.29	44.24–93.73	2.84 ± 0.55	25.99	0.94
7	NBAIR Bt114	47.03	32.19–66.81	2.72 ± 0.53	25.71	0.96
8	NBAIR Bt119	65.5	44.64–94.69	2.85 ± 0.56	25.36	0.89
9	NBAIR Bt120	54.65	16.50–58.65	1.59 ± 0.48	10.94	0.67
10	NBAIR Bt142	39.83	25.03–57.91	2.35 ± 0.52	20.03	0.95
11	Bti (4Q1)	31.3	18.46–45.35	2.24 ± 0.52	17.94	0.62

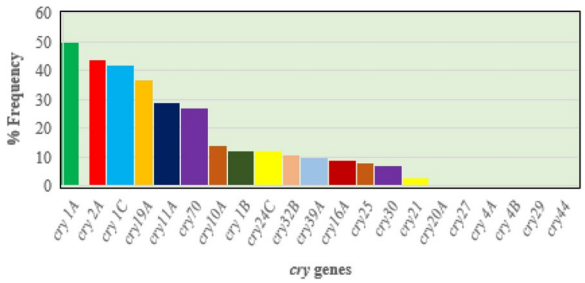

Fig. 2 The frequency distribution of *cry* genes in indigenous *Bacillus thuringiensis* strains

combinations were more frequently observed among strains than any other gene combination, as 88% (44/50) of strains were positive for both genes. So, *cry1A*, *cry1C* and *cry2* were the most commonly found *cry* genes in indigenous *Bt* strains, and these results are consistent with other reports (Zhang et al. 2000). Among the 21 dipteran active *cry* genes evaluated in the current study, *cry1A*, *cry 1C*, *cry2A*, *cry10*, *cry11*, *cry19* and *cry70* were found to be more frequent in the native *Bt* strains.

Looking into the *cry* gene profiles of first groups of strains, NBAIR 101 had *cry1 A-C*, *cry2A*, *cry10*, *cry11*, *cry19*, *cry25* and *cry70*, whereas NBAIR Bt104 also had a similar profile, except for *cry19,* but had a *cry24*. Various studies proved that strains with *cry4*, *cry10*, *cry11* and *cyt* gene combinations, highly effective against mosquitoes (Ibarra et al. 2003) and also that high toxicity of strains due to the synergetic action of *cry4* and *cry11* is reported (Hayakawa et al. 2017). Similarly, the gene *cry19* has been reported to increase the toxicity of strains in combinations with the *orf2* gene, which is reported to have sequence similarity with *cry4B,* and this combo is similar to the operon structure of the *cry10-orf2* format reported in *Bti* (Rosso et al. 1997). Beron and Salerna (2007) in *cry24* family, *Cry24Ca1* was reported to have high toxicity against *Aedes aegypti* Linnaeus (Diptera: Culicidae). In the same way, a study conducted by Fayad and co-workers showed novel *cry* genes, such as *cry70B,* reported to have an anti-dipteran activity (Fayad et al. 2021). Based on the present study, we may predict that *cry* genes such as *cry10*, *cry11* and *cry19* might interact with other *cry* genes present in a strain and these genes are probably interacting with other *cry* and *cry70* as both the strains tested negative for *cry4A* and *cry4B* genes.

The *cry* gene profiles of strains NBAIR Bt111 and NBAIR Bt121 were found to be similar to those of NBAIR Bt101 and NBAIR Bt104, except for the gene *cry10*. It shows that *cry10* may be responsible for enhanced toxicity in NBAIR Bt101 and NBAIR Bt104. The presence of *cry16* and *cry27* in strain NBAIR Bt111 may be responsible for more toxicity compared to NBAIR Bt121, as the *cry16* gene, when co expressed with other genes, was found to cause increased toxicity (Qureshi et al. 2014), and *cry27* also caused toxicity to *Anopheles stephensi* Listen (Diptera: Culicidae) (Saitoh et al. 2000).

Among the second group of strains that harbored eight dipteran active *cry* genes in different combinations, only the two strains NBAIR Bt114 and NBAIR Bt142 showed a combination of the *cry10, cry11*, *cry*19 and *cry70* genes. Among the second group of strains that harbored eight dipteran active *cry* genes in different combinations, three strains, NBAIR Bt113, NBAIR Bt114 and NBAIR Bt142, the *cry* gene profiles of these two strains were very similar, and these three were the only strains that showed a combination of *cry1A-C*, *cry2A*, *cry10, cry11*, *cry*19 and *cry70*. This shows that interaction among these genes causes maximum mortality of melon fruit fly larvae. Similarly, among the strains that were positive for either seven, six, five, four or and three *cry* genes, only three strains, such as NBAIR Bt107, NBAIR Bt119 and NBAIR Bt120, caused the highest mortality. The *cry* gene profiles

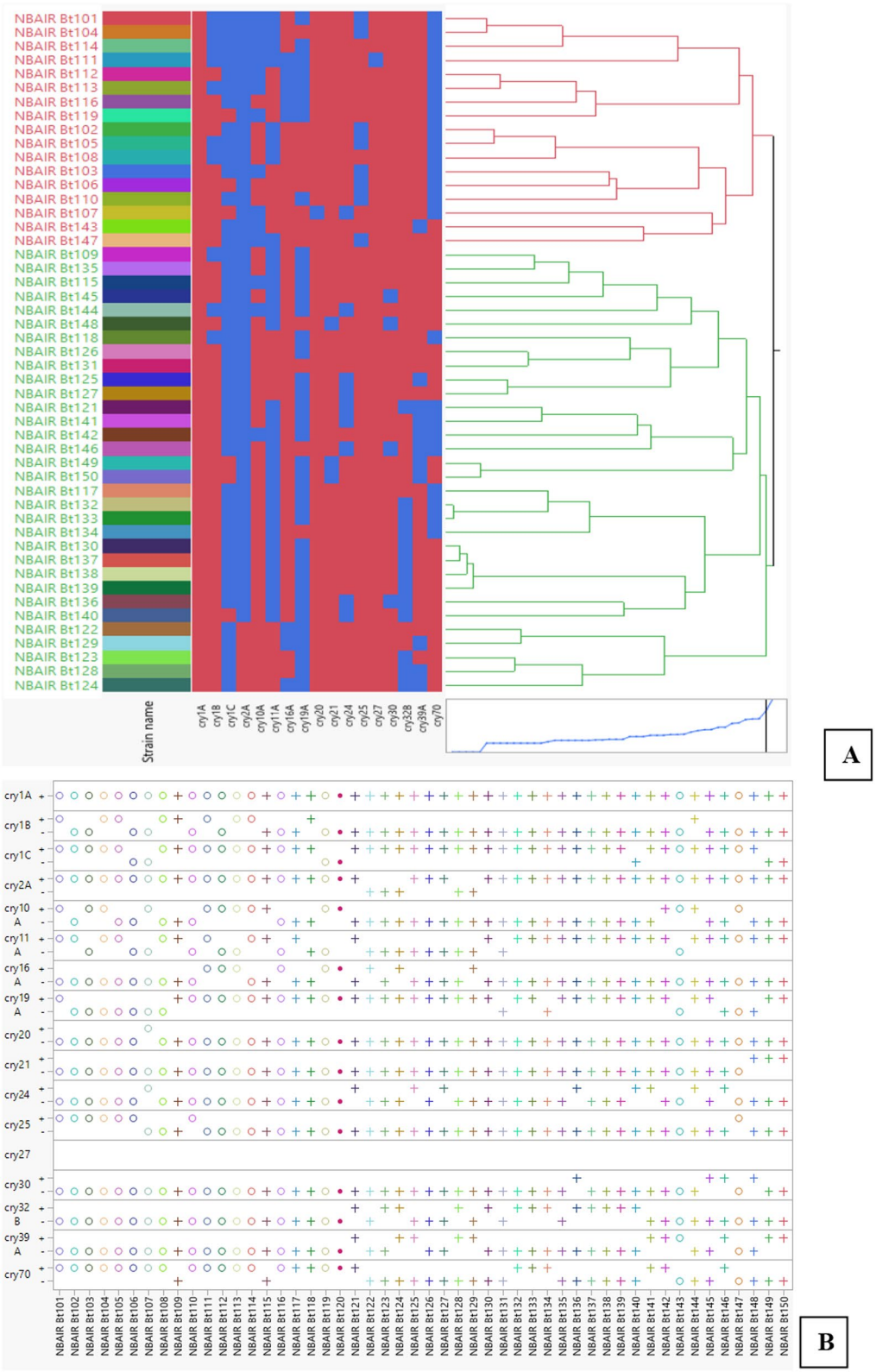

Fig. 3 **A** Dendrogram showing the *cry* gene profile of fifty indigenous *Bacillus thuringiensis* strains. **B** Scatter plot matrix of *cry* gene *vis-a-vis* to indigenous *B. thuringiensis* strains

of these strains revealed that *cry10* or *cry70* was the most common *cry* genes that were found. The strains caused more than 50% mortality had a combination of *cry1A*, *cry2A*, *cry10A*, *cry70*. Moreover, the strains that are negative for either *cry10* or *cry70* showed a very low mortality compared to strains that were positive for both strains. However, further research has to be conducted on certain aspects, like cloning the *cry1A*, *cry2A*, *cry 10A* and *cry70* to be done individually to evaluate the potential toxicity and synergistic activity with other *cry* genes of interest.

Interestingly, even though strain NBAIR Bt107 harbored only six dipteran active *cry* genes, it is the only strain that tested positive for *cry20*. In contrast, NBAIR Bt142 was almost equal to NBAIR Bt107, but instead of *cry20*, it had *cry39*. Similarly, strains such as NBAIR Bt112, NBAIR Bt113 and NBAIR Bt119 had more or less equal LC_{50} values, and all these strains were tested positively for *cry16* and *cry19,* apart from *cry 10* and *cry70*. NBAIR Bt114 strain tested positive for *cry11* instead of *cry16*. Despite the similarity in mortality percentage and *cry* gene profiles, differences in LC_{50} were found between two strains, NBAIR Bt101 and NBAIR Bt104. This shows that not the greater number of *cry* genes and its mere presence of *cry* genes; expression levels of *cry* genes determine the toxicity. However, further studies have to be carried out to study the role of individual *cry* genes and the combination of *cry* genes causing mortality in larvae of *Z. cucurbitae*.

Conclusion

The frequency and distribution of dipteran active *cry* genes were detected in fifty indigenous *Bt* strains. All the strains tested positive for more than one *cry* gene. Ten out of 50 *Bt* strains displayed high toxicity against *Z. cucurbitae*. Among the *cry* genes, *cry1A*, *cry1B*, *cry1C*, *cry2A*, *cry10A*, *cry11A*, *cry16A*, *cry19A*, *cry20*, *cry24, cry25, cry39A* and *cry70* were detected in high frequency. The probable *cry* genes responsible for causing toxicity in larvae were interpreted based on a comparison of the *cry* gene profiles of each strain and corresponding larval mortality. The common combination in all ten strains that displayed great toxicity included *cry1A*, *cry2A*, *cry10A* and *cry70*. Hence, this study paves the way for further studies in testing the toxicity of individual *cry* gene or interactions among genes and thus helps in identification of potential *cry* genes responsible for causing mortality. So, effective strains with potential *cry* genes can be used for the management of *Z. cucurbitae*. This further helps the development of an environmentally friendly bioformulation in anticipation of its possible application in the field and developed of *Bt*-based transgenic crops.

Abbreviations

Bt	*Bacillus thuringiensis*
hrs.	Hours
^0C	Degree Celsius
RH	Relative humidity
LC_{50}	Median lethal concentration
µg	Microgram
min	Minutes
cry	Crystal
NCBI	National Centre for Biotechnology Information
mL	Milliliter

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s41938-024-00811-6.

Additional file 1. Agarose gel electrophoresis of PCR products amplified with dipteran active cry genes in indigenous *Bacillus thuringiensis* strains.

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Author contributions

CM, AN, ANS and VKD conceptualized the research. AN conducted experiment. AK, AK and RR monitored experiments and edited the manuscript. All the authors have read the manuscript and approved for submission.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All procedures performed in the study are in accordance with the ethical standard of the institutional and/or national research committee. We further declare that no animal was harmed during the study.

Consent for publication

Informed consent was obtained from all the individual participants included in the study.

Competing interest

The authors declare that there is no conflict of interest.

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