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Molecular Characterisation and Toxicity Analysis of Indigenous *Bacillus thuringiensis* Berliner Isolates against Cucurbit Fruit Fly Maggots, *Zeugodacus cucurbitae* (Coquilett) (Diptera: Tephritidae)

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ABSTRACT

Background: Indiscriminate use of insecticides against fruit flies (Tephritidae: Diptera) has led to the development of residue and resistance and there is a need to try alternate eco-friendly management practices. *Bacillus thuringiensis* (Bt) is the most widely used bacterium forming proteins active against insects of different orders. Thus, the present study was framed to characterize and evaluate 50 indigenous Bt isolates against cucurbit fruit fly maggots, *Zeugodacus cucurbitae.*

Methods: Colony morphology was observed visually, while crystal morphology was observed through a microscope. The *cry* gene content of the Bt isolates was screened by Polymerase Chain Reaction and the proteins harbored were assessed by SDS- PAGE analysis. Full diet contamination method was used to evaluate the toxicity of Bt isolates against cucurbit fruit fly maggots.

Result: Uniform full white, off-white and creamy white colony colours were found except one isolate each, exhibiting full white at centre surrounded by off white and off white at centre surrounded by full white. Spherical shape crystal (45.10%) was predominant followed by cuboidal (29.41%), bipyramidal (17.65%), rectangular (3.92%) and minute crystal attached to spore (3.92%). Insecticidal proteins varied from ~15 to >200kDa in size with one to four and more distinct bands per isolate. PCR screening revealed *cry4Aa* in 2 isolates, *cry4Ba* and *cry11Aa* in one isolate each, *cyt1* in 1 isolate. The standard strain Bti 4Q2 (100% mortality of maggots) and the indigenous Bt isolates, T166 (92.59%), T184 (91.11%) and T60 (90.48%) were found to be significantly toxic.

Key words: Artificial diet contamination, *Bacillus thuringiensis,* Diversity, Insecticidal genes, Melon fly maggots, Proteins, Toxicity.

INTRODUCTION

Cucurbits occupy 66 percent of the vegetable land in India as commercial and kitchen garden crops, producing only 11 per cent of the total vegetable production (Nasiruddin *et al*., 2004). The genus *Bactrocera* with more than 40 species is the major constraint, among the 200 species of Tephritidae listed in India (Kapoor, 2002). Among which, the cucurbit fruit fly/melon fruit fly, *Zeugodacus* (*Bactrocera*) *cucurbitae* (Coquillett) causes 30 to 100 per cent loss (Dhillon *et al*., 2005).

With the increasing awareness among farmers for organic cucurbit production, cucurbit fruit flies have been managed with several eco-friendly practices (Ansari *et al*., 2012; Nelson, 2019), but they are uneconomical with 25 per cent of the production cost spent solely to manage cucurbit fruit fly (Nasiruddin *et al*., 2004). *Bacillus thuringiensis* Berliner (Bt) of the family Bacillaceae forms resistant spores of proteins against insects of different orders (Schnepf *et al*., 1998). This remains a possibility and also imposes the need for the search of native Bt isolates with activity against the cucurbit fruit fly, *Z. cucurbitae.*

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MATERIALS AND METHODS

Laboratory maintenance of insect culture

Laboratory culture of *Z. cucurbitae* was established initially from TNAU orchard (11°0′31″N latitude: 76°55′39″E longitude) from the fruit fly infested cucurbits showing symptoms such as distorted shape and oozing of resinous fluid (Lal *et al*., 2014). The freshly emerged adult flies from the infested cucurbits were identified as the cucurbit fruit fly, *Z. cucurbitae* based on its taxonomic characters (Prabhakar *et al*., 2012), sexed (Mir *et al*., 2014) and released into cages (42 cm length × 42 cm width × 42 cm height). Cucumber slices (ca. 1mm thickness) were cut and placed in piles of 3-4 slices in petri dishes and kept inside cages with adults for oviposition. The cucumber slices in piles were replaced every 24 hours for fresh oviposition (Liu *et al*., 2020). Adults were fed with 10 percent sugar solution suspended with vitamin E through a piece of soaked clean cotton wick placed inside a glass vial (2.3 cm diameter × 5.3 cm height). The cucumber slices in petri dishes with eggs were placed in plastic boxes for the development of maggots. Cucumber slices were replaced or added when necessary and the maggots were fed *ad-lib.* throughout the study. As the maggots reached the third instar and were about to pupate, the plastic boxes were transformed in to trays containing sand of 2 cm height to facilitate pupation. Pupae were sieved from the sand after 2 days and placed inside cages in petri dishes for adult emergence. The insect culture was maintained under controlled conditions of temperature, humidity and photoperiod (25±2°C, 70±10% RH and 12 L: 12D) at the insect bioassay laboratory (Department of Plant Biotechnology, TNAU, Coimbatore, India) and was reared for ten successive generations before initiating bioassay experiments.

Bt isolates and growth conditions

Indigenous Bt isolates (n=50) and negative Bt check (4Q7) were obtained from the Bt laboratory, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India. The standard reference strain of *Bacillus thuringiensis* subsp. *israelensis* used in the study as positive check was obtained from Bacillus Genetic Stock Centre (BGSC, Columbus, Ohio) (Supplementary Table 1). The bacterial cultures were revived, sub-cultured and stored for further use (Ramalakshmi and Udayasuriyan, 2010).

Characterisation of bacterial colony and crystal morphology

Colony shape, surface, colour, margin and elevation of individual Bt isolates were examined. Crystals were documented for their morphology using Leica (DM 1000LED, DFC295, Germany) microscopy (Sharif and Alaeddinoglu, 1988; Ramalakshmi and Udayasuriyan, 2010).

Spore crystal mixture isolation from Bt isolates

Spore crystal mixture was isolated based on the method described by Ramalakshmi and Udayasuriyan (2010).

Characterization of protein

The SC mixtures were analysed for the presence of δ-endotoxins by SDS-PAGE (Sodium dodecyl sulphate poly acrylamide gel electrophoresis) (Laemmli, 1970). Pre stained three colour protein marker (Puregene, Genetix Biotech Asia Pvt Ltd.,) which spans wide range of molecular weights from 10 to 315 kDa was used to document the molecular weight of the proteins.

Insecticidal gene profiling of Bt isolates

In Bt cultures, genomic DNA was extracted using the technique outlined by Kalman *et al*. (1993). Polymerase chain reaction (PCR) was performed (20 µl) with gene-specific primers for the genes *cry4Aa, cry4Ba, cry10Aa, cry11Aa* and *cyt1*. The amplification conditions were programmed according to the specifications mentioned in Supplementary Table 3. The amplified products were observed for the expected size of amplicon of each gene (Supplementary Table 2) (Ben-Dov *et al*., 1997).

Toxicity of Bt isolates against cucurbit fruit fly maggots

ELISA reader (Biotek-Powerwave XS) was used to estimate the concentration of protein in SC mixtures by Broadford's reagent method (He, 2011). Artificial diet was used to evaluate the toxicity of SC mixtures against cucurbit fruit fly, *Z. cucurbitae* maggots. The proportion of various ingredients used in preparation of artificial diet (AD) is 2.52g pumpkin, 8.84 g common bean powder, 2.27g yeast extract powder, 3.53 g sugar (sucrose), 0.63 g agar, 0.21 g vitamin E capsule, 200 µl Zincovit and 100 ml of sterile distilled water (composition for 100ml of AD). Agar and other homogenized ingredients (pumpkin, common bean powder, yeast extract and sucrose) were boiled in separate containers, mixed together, allowed to cool, then vitamins were added, blended again and distributed into containers.

Supplementary Table 1: Details of indigenous Bt isolates and reference strains used in the study.

Description	Details of Bt
Positive control for *cry4Aa*, *cry 4Ba*, *cry 10Aa*, *cry11Aa* and *cyt1* genes	*Bacillus thuringiensis* subsp. *israelensis* HD500 (Bti) (BGSC No. 4Q2)
Negative control	4Q7
Indigenous Bt isolates	T56, T58, T59, T60, T61, T62, T63, T67, T69, T71, T74, T76, T77, T78, T79, T81, T82, T83, T84, T86, T87, T88, T89, T90, T91, T93, T94, T95, T96, T97, T162, T164, T165, T166, T167, T168, T172, T173, T174, T176, T178, T179, T181, T183, T184, T185, T186, T187, T188, T189

Bioassay was conducted using different concentrations of protein (0.03 to 2.3 µg of protein/µl of SC mixture isolated) with the standard reference strain Bti 4Q2. Based on the result, the estimated protein concentration of indigenous Bt isolates was equalised to a concentration of 0.7 µg/µl and the toxicity was evaluated by whole diet contamination (Aboussaid *et al*., 2010) by mixing 150 µl of quantified SC mixture to 500 µl of AD solidified (the maggots were fed *ad lib.* throughout the experiment) in plastic cups (2.3 cm height × 3 cm diameter) with a glass rod under sterile conditions. To perform *in vitro* bioassay, the *Z. cucurbitae* eggs (less than 24 h old) of the same cohort were collected from the piles of cucumber slices removed from the cages after 12 hours of exposure to oviposition. Eggs were removed using fine brush and placed over fresh cucumber slices. Maggots newly emerged from the eggs were harvested by placing the cucumber slices in sterile distilled water (Kaur *et al*., 2010). The maggots were released into plastic cups with AD contaminated with SC mixture using a fine camel hair brush without any physical damage. Three replications were maintained (10 maggots/replication). Observation was made for 7 days. Standard strain, Bti 4Q2 was the positive check, acrystalliferous strain 4Q7 was the negative check and water was used as control (Ilias *et al*., 2013).

Statistical analysis

The experimental design was completely randomized (CRD). The cumulative mortality was expressed in percentage, computed using formula (Abbott, 1925) when the mortality in control exceeded 5 percent but was less than 20 per cent. The data was analysed by one way ANOVA using AGRES (version 7.01). Least significant difference (LSD) was used to determine the statistical significance. The research was carried out from 2020 to 2022.

RESULTS AND DISCUSSION

Colony and crystal morphology of Bt isolates

Colony colour was observed as full white (15.38%), creamy white (11.54%) and off white (69.23%). One isolate each was found to express full white colour at the centre encircled by off white and creamy white colour at the centre encircled by off white (Table 1). Navya *et al.* (2021) have reported the predominant occurrence of off-white colonies (53 isolates) followed by full white (7 isolates) favoring the present findings. Occurrence of creamy white colonies is accorded by Gothandaraman *et al.* (2022). The colony surface was observed to be glossy (9.62%), fried egg (40.38%) and smooth (50%). Circular (37 isolates) and irregular shaped (15) colonies were observed. Elevation was flat (55.77 %) and raised (44.23%). Colony margins appeared entire (22 isolates) and undulate (30) (Table 1). Colony type was observed as fried egg/mucoid/smooth with raised and flat elevation with entire/undulate margins and appeared in circular/ irregular shapes (Navya *et al*., 2021; Gothandaraman *et al*., 2022) in line with the present findings. Crystal morphology revealed the dominant occurrence of spherical crystals (45.10%) followed by cuboidal (29.41%) and bipyramidal (17.65%) (Table 2). This is supported by the occurrence of spherical crystals in 99

Supplementary Table 2: Details of primers used in the study for screening indigenous Bt isolates.

cry gene	Primer sequence	Amplicon size (bp)	Reference
cry4Aa	FP: 5′-GAACTGGGTATGGCACTCAAC-3′	~777	Soares da silva *et al.,* (2017)
	RP: 5′-CTCACAACGATTAGACCCTTC-3′		
cry4Ba	FP: 5′-GCGAGGTTTCCCATGTCTAC-3′	~347	
	RP: 5′-GTTGTAGGGTGGAATTGTTATC-3′		
cry10Aa	FP: 5′-ATTGTTGGAGTTAGTGCAGG-3′	~995	
	RP: 5′-AATACTTTGGATGTGTCTTGAG-3′		
cry11Aa	FP: 5′-AGGATGGATAGGAAACGGAAG-3′	~727	
	RP: 5′- CCGTATTCCAGCAGGTAAGC-3′		
cyt1	FP: 5′-CCTCAATCAACAGCAAGGGTTATT-3′	~477-	Ibarra *et al.* (2003)
	RP: 5′-TGCAAACAGGACATTGTATGTGTAATT-3′	489	

Supplementary Table 3: PCR conditions for screening Bt isolates for specific genes.

Step	Temperature profile and time				
	cry4Aa	*cry4Ba*	*cry10Aa*	*cry11Aa*	*cyt1*
Initial denaturation	94°C for 5 minutes				94°C for 2 minutes
Denaturation	94°C for 30 seconds		94°C for 45 seconds		
Annealing	60°C for 50 seconds		60°C for 45 seconds		52°C for 45 seconds
Extension	72°C for 45 seconds		72°C for 50 seconds		72°C for 45 seconds
Step 2 to 4	38 cycles				35 cycles
Final extension	72°C for 10 minutes				

Table 1: Colony morphology of Indigenous Bt isolates.

	Colony parameters	Occurrence (%)
Colour	Full white	15.38
	Creamy white	11.54
	Off-white	69.23
	Full white encircled by off white	1.92
	Creamy white encircled by off white	1.92
Surface	Fried egg	40.38
	Smooth	50.00
	Glossy	9.62
Shape	Irregular	28.85
	Circular	71.15
Elevation	Raised	44.23
	Flat	55.77
Margin	Entire	42.31
	Undulate	57.69

Table 2: Crystal morphology of Indigenous Bt isolates.

Crystal shape	Occurrence (Number)	Occurrence (%)
Bipyramidal	9.00	17.65
Cuboidal	15.00	29.41
Spherical	23.00	45.10
Crystal attached to spore	2.00	3.92
Rectangular	2.00	3.92
Acrystalliferous	Negative control	4Q7

Table 3: Insecticidal protein distribution in Indigenous Bt isolates from SDS-PAGE analysis.

Protein size (kDa)	Occurrence (Number)	Occurrence (%)
>200	9	17.65
~171	1	1.96
~134	8	15.69
~124	2	3.92
~110	4	7.84
~100	7	13.73
~95	6	11.76
~80	3	5.88
~70	36	70.59
~65	8	15.69
~55	28	54.90
~45	30	58.82
~30	19	37.25
~26	18	35.29
~15	4	7.84
Protein bands (No.)	Occurrence (Number)	Occurrence (%)
One	6	11.76
Two	9	17.65
Three	9	17.65
Four and more	27	52.94

per cent of the isolates against mosquitoes (Mahalakshmi *et al*., 2012).

Protein profiles of Bt isolates

Insecticidal proteins with varying molecular weights ranging from ~15 to >200 kDa were found. The banding pattern was observed to have one band (11.76%), two to three (17.65%) and four or more bands (52.94%) (Table 3). Diversified protein patterns having molecular weights between ~20 and more than 200 kDa and variable number of protein bands above one have been reported earlier (Navya *et al*., 2021; Gothandaraman *et al*., 2022) in line with the current study.

Distribution of dipteran-specific insecticidal genes in Bt isolates

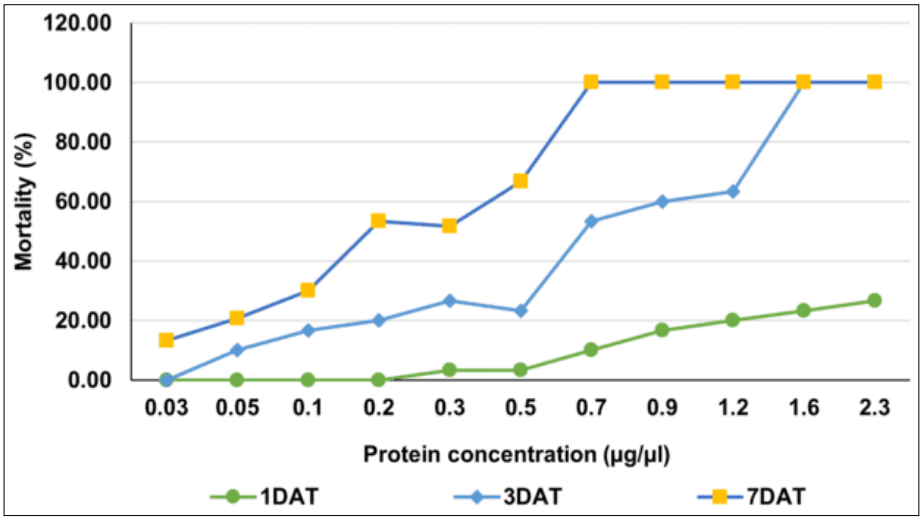
PCR screening was done for five dipteran-specific genes (*cry4Aa, cry4Ba, cry10Aa, cry11Aa* and *cyt1*). Two isolates *viz*., T166 and T184 were positive for *cry4Aa*. The isolate T60 was positive for *cry4Ba* and the isolate T166 was positive for *cry11Aa* gene. Among the 50 isolates screened, the gene *cyt1* was positive in 1 isolate (T166). The remaining 47 number of indigenous Bt isolates did not show the presence of these five genes screened. No isolate showed PCR positive result for the gene *cry10Aa*. One isolate, T166 was positive for three genes *viz*., *cry4Aa, cry11Aa* and *cyt1* in combination (Table 4). Jain *et al.* (2017) reported diverse *cry* gene profiles with the occurrence of *cry4* (84.14%) and *cry11* (39.28%) genes. Genes *viz*., *cry 4A, cyt 1, cry 10, cry 11* and *cry 4B* were found in *B. thuringiensis* strain B474 (Geetha *et al*., 2007).

Toxicity of Bt isolates against *Z. cucurbitae* maggots

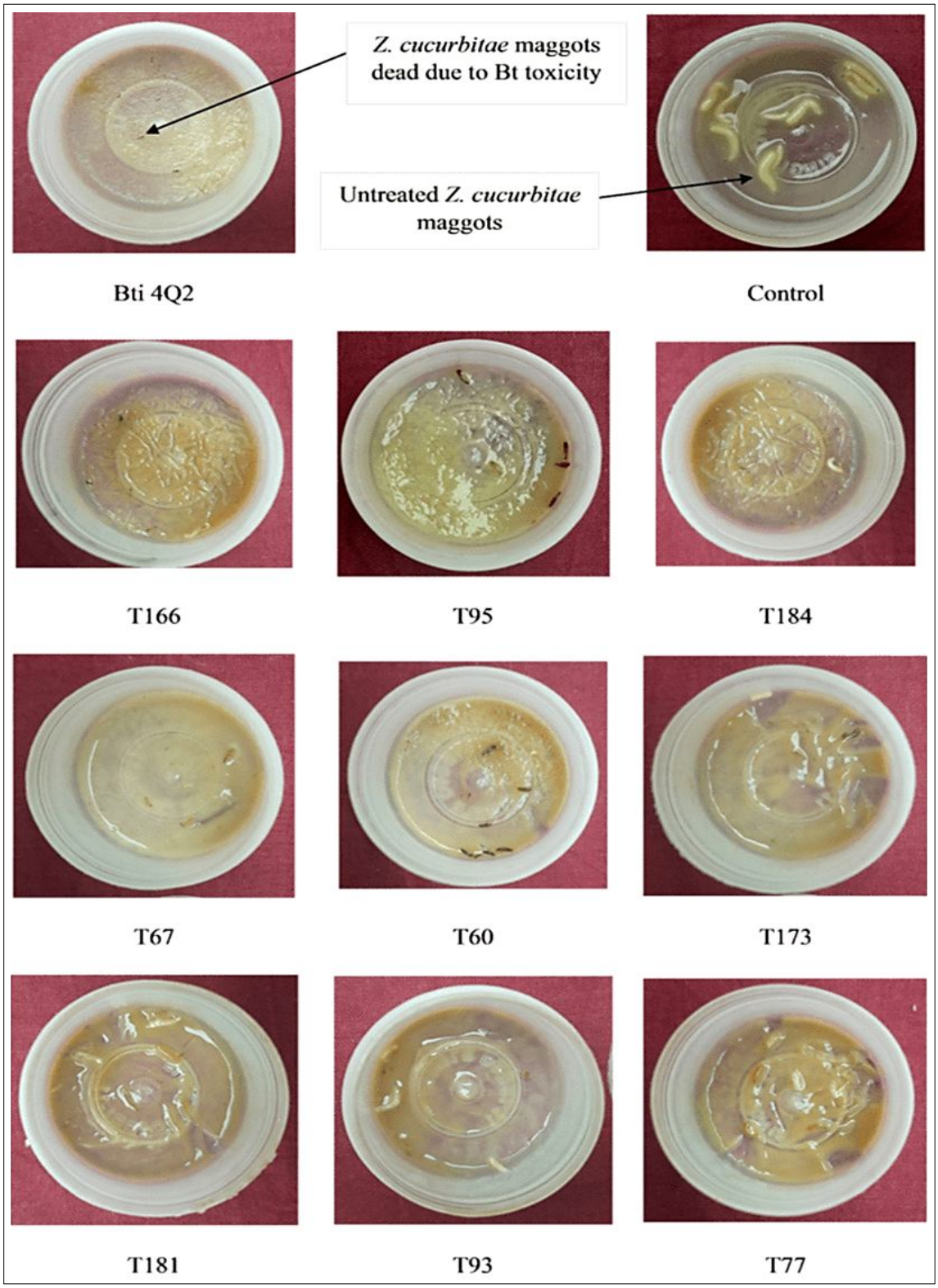
Observations on insecticidal activity of Bti 4Q2 at different concentrations revealed, 100 percent mortality of maggots at an initial concentration of 0.7µg/µl at seventh day after treatment (DAT) (Supplementary Fig 1). Three indigenous Bt isolates *viz*., T60, T166 and T184 produced 90.48, 92.59 and 91.11 percent mortality respectively (Table 5) (Supplementary Fig 2). Bt strain from biotype *kurstaki* (96%) and the indigenous Bt JSc 1 (93%) harbouring *cry1A* type genes exhibited higher toxicity on 3rd instar *Z. cucurbitae* maggots (Shishir *et al*., 2015). Bt 13.4 toxin exhibited mortality (100 %) against Mediterranean fruit fly, *Ceratitis*

Table 4: Insecticidal gene distribution in indigenous Bt isolates from PCR screening.

Insecticidal gene	Occurrence (Number)	Occurrence (%)
cry4Aa	2	4.00
cry4Ba	1	2.00
cry10Aa	0	0.00
cry11Aa	1	2.00
cyt1	1	2.00
cry4Aa + cry411Aa + cyt1	1	2.00
cry4Aa + cry4Ba + cry10Aa + cry411Aa + cyt1	Standard reference strain	*Bacillus thuringiensis* subsp. *israelensis* HD500 (BGSC No. 4Q2)



Supplementary Fig 1: Toxicity of *Bacillus thuringiensis* subsp. *israelensis* (HD 500) at different concentrations of insecticidal protein(s) against cucurbit fruit fly maggots.



Supplementary Fig 2: *In vitro* insect bioassay with spore-crystal mixtures of Bt isolates against neonate cucurbit fruit fly, *Zeugodacus cucurbitae* maggots showing difference in mortality and feeding of artificial diet on 7 days after treatment.

Table 5: Toxicity of indigenous Bt isolates against maggots of cucurbit fruit fly, *Z. cucurbitae.*

Bt isolates	Per cent mortality of maggots at 7 DAT (0.7 µg/µl)
T56	42.13 (40.47)^{fghijkl}
T58	7.41 (15.80)^{opqr}
T59	17.41 (24.66)^{lmnopqr}
T60	90.48 (72.03)^{ab}
T61	17.50 (24.73)^{jklmnopq}
T62	15.00 (22.79)^{lmnopqr}
T63	10.37 (18.79)^{nopqr}
T67	85.19 (67.64)^{abc}
T69	22.17 (28.09)^{jklmnpq}
T71	37.30 (37.64)^{ghijklm}
T74	20.74 (27.09)^{klmnopq}
T76	26.20 (30.79)^{ijklmnop}
T77	72.01 (58.06)^{bcdefg}
T78	49.91 (44.95)^{efghij}
T79	18.70 (25.62)^{jklmnopq}
T81	18.24 (25.28)^{jklmnopq}
T82	29.44 (32.86)^{hijklmno}
T83	26.57 (25.28)^{ijklmnop}
T84	7.04 (15.39)^{opqr}
T86	11.20 (19.55)^{lmnopq}
T87	14.07 (22.03)^{mnopqr}
T88	21.11 (27.35)^{klmnopq}
T89	13.70 (21.72)^{mnopqr}
T90	3.33 (10.51)^{qr}
T91	0.00 (0.40)^{r}
T93	76.39 (60.93)^{bcdef}
T94	48.04 (43.88)^{fghijk}
T95	80.00 (63.44)^{abcd}
T96	68.52 (55.87)^{bcdef}
T97	21.48 (27.61)^{klmnopq}
T162	7.41 (15.80)^{pqr}
T164	7.50 (15.89)^{opqr}
T165	3.70 (11.09)^{qr}
T166	92.59 (74.21)^{ab}
T167	56.94 (48.99)^{defghi}
T168	19.76 (26.39)^{jklmnopq}
T172	15.28 (23.01)^{klmnopq}
T173	79.37 (62.99)^{cde}
T174	40.00 (39.23)^{gijklm}
T176	65.74 (54.18)^{cdefgh}
T178	22.69 (28.45)^{ijklmnopq}
T179	10.37 (18.79)^{nopqr}
T181	77.50 (61.69)^{bcdef}
T183	24.44 (29.63)^{jklmnopq}
T184	91.11 (72.65)^{abc}
T185	34.34 (35.87)^{hijklmn}
T186	0.00 (0.40)^{r}
T187	17.78 (24.94)^{lmnopqr}
T188	65.28 (53.90)^{cdefgh}
T189	28.15 (32.04)^{jklmnopq}
Bti 4Q2	100.00 (89.61)^{a}
4Q7	0.00 (0.40)^{r}
Control	0.00 (0.40)^{r}
SEd	11.11
CD (p=0.05)	22.03

Table 5: Continue...

Table 5: Continue...

Figures in parentheses are arc sine transformed values of percentage.

Values followed by the same letters in a column are not significantly different (p=0.05).

Bti 4Q2 is the standard strain.

capitata (Wiedemann) neonate maggots 7 days after exposure, in accordance with the present result (Aboussaid *et al*., 2010).

CONCLUSION

Among the 50 indigenous Bt isolates evaluated, three were highly toxic with more than 90 per cent mortality of cucurbit fruit fly maggots. Insecticidal activity was found to persist in few isolates despite the absence of five genes screened in the present study, but with the expression of distinct protein bands coded by other genes.

Conflict of interest

The authors declare that they have no conflict of interest.

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