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TOXICITY OF *BACILLUS THURINGIENSIS* ISOLATES TO THE CUCURBIT FRUIT FLY *ZEUGODACUS CUCURBITAE*

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ABSTRACT

The management of melon fly *Zeugodacus cucurbitae* (Coquilett) having wide host range is most challenging. *Bacillus thuringiensis* remains a potential pest management candidate of biological origin. In the present study, nine indigenous Bt isolates were characterised and evaluated against neonate (~12 hrs old) maggots. The isolates showed a diversified nature of colony and crystal morphology. Concentration-response toxicity assay revealed that three isolates viz., T166, T60 and T184 were highly toxic with LC_{50} values of 0.38, 0.41 and 0.40 µg/ µl, respectively on par with the reference strain Bti 4Q2 with 0.37 µg/ µl. LC_{95} of Bt cultures ranged from 1.15 to 2.09 µg/ µl. Gene profiling revealed the occurrence of *cry4Aa* in T166 and T184, *cry4Ba* in T60, *cry11Aa* in T166 and *cyt1* in T166 with protein profiling showing proteins of ~134, ~128, ~72 and ~27kDa, respectively.

Key words: Bt cultures, colony features, crystal shape, PCR, cry gene, cyt gene, SDS PAGE, insecticidal proteins, concentration-response, bioassay, whole diet contamination, LC_{50}, LC_{95}

A variety of cucurbit crops are cultivated extensively in India (Seshadri and More, 2009) and diverse group of insect pests have been reported to constrain the production and productivity. Among them, cucurbit fruit fly/ melon fly *Zeugodacus cucurbitae* (Coquilett) has been estimated to damage flowers and fruits causing 20 to 100% loss (Saptoka et al., 2010; Haider and Rai, 2021). Cucurbits are harvested at short intervals for marketing and self-consumption before the waiting period of insecticides. Furthermore, male and female flowers of cucurbits occur separately as staminate and pistillate flowers necessitating an ecofriendly management practice favouring insect pollinators (Dorjay and Abrol, 2022). In addition, with an increasing awareness of organic farming, India holds largest number of organic producers (Willer et al., 2024), necessitating the shift from insecticides to management practices ideal for organic farming system. Biocontrol agents such as parasitoids, fungus, and nematodes were the most investigated against fruit flies than predators, bacteria and viruses (Diksha et al., 2022); *Bacillus thuringiensis* Berliner was the most studied bacterium among the eleven bacterial species evaluated as biocontrol agents of fruit flies with *Z. cucurbitae* as one of the main fruit fly hosts (Dias et al., 2022); but this has not been principally exploited for the management of *Z. cucurbitae*. With this in view, the present study was conducted to characterise and evaluate the efficacy of indigenous Bt isolates toxic to *Z. cucurbitae* maggots.

MATERIALS AND METHODS

Insect culture was maintained and reared based on the method described by Sharmitha et al. (2024). Nine indigenous Bt isolates (T60, T67, T77, T93, T95, T166, T173, T181, T184) were obtained from the Bt laboratory, Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India and research was conducted from 2022 to 2023. The standard reference strain of *Bacillus thuringiensis* subsp. *israelensis* HD 500 (Bti) (BGSC No. 4Q2) was obtained from Bacillus Genetic Stock Centre (BGSC, Columbus, Ohio). The isolates were further maintained and cultured on T3 for visual and microscopic (Leica, DM 1000LED, DFC295, Germany) examination of colony (shape, surface, colour, margin and elevation) and crystal morphology respectively (Ramalakshmi and Udayasuriyan, 2010; Sharif and Alaeddinoglu, 1988). The genes *cry4Aa, cry4Ba, cry10Aa, cry11Aa* and *cyt1* encoding dipteran toxic proteins were screened by Polymerase Chain Reaction (PCR) using bacterial genomic DNA isolated based on Kalman et al. (1993). The details of the primers used in the study and amplification conditions are according to the specifications mentioned in Sharmitha et al. (2024). Amplified PCR product was electrophoresed and imaged in a gel documentation unit (Bio-print, Vilber). SDS-PAGE (Sodium dodecyl sulphate poly acrylamide gel electrophoresis) was run according to Laemmli (1970) to document the protein profiles of crude spore crystal (SC) mixtures isolated (Ramalakshmi and Udayasuriyan, 2010) using protein marker (10 to 250 kDa range) as reference. The concentration of insecticidal protein in the SC mixture was estimated by Bradford's reagent method (He, 2011) and diluted to various concentrations *viz.,* 0.03, 0.1, 0.2, 0.5, 0.7, 0.9, 1.2, 1.6 and 2.3 µg/ µl for toxicity analysis. Concentration-response bioassay was conducted based on the artificial diet and protocol specifications reported by Sharmitha et al. (2024). Three replications were maintained with 10 maggots/ replication in a completely randomised design (CRD). The standard strain Bti 4Q2 was used as positive check and sterile distilled water was used as control. Maggot mortality was observed at 24 hrs intervals for 7 consecutive days. Abbot's formula was used when the mortality in control exceeded 5% but was less than 20% (Abbott, 1925). The cumulative mortality on the 7th day after treatment was computed and used to calculate the median lethal concentration (LC_{50}). Statistical analysis for calculating LC_{50} and LC_{95} values for each bacterial culture was performed based on Finney's method of probit analysis (Srinivasan et al., 2017).

RESULTS AND DISCUSSION

The LC_{50} values of Bt isolates ranged from 0.37 to 0.52 µg/ µl and LC_{95} ranged from 1.15 to 2.09 µg/ µl. Bt isolates T166, T60 and T184 were highly toxic to fruit fly maggots with LC_{50} values of 0.38, 0.41and 0.40 µg/ µl, respectively as compared to 0.37 µg/ µl in Bti 4Q2 with the same trend in LC_{95} values. The fiducial limits of the isolates T60, T166 and T184 were observed to overlap with the standard strain Bti 4Q2 confirming the significant similarity in the toxicity exhibited (Table 1; Fig. 1). Previously, indigenous Bt strains JSc1, SSc2 (Shishiri et al., 2015), Btk HD-73 (Bari et al., 2021) and NBAIR Bt 107 (Aarthi et al., 2024) were reported to be toxic to *Z. cucurbitae* on par with standard check from different parts of India. Variation in colony and crystal morphology was evident among the nine isolates (Table 2) as reported earlier (Ganga and Arjyal, 2020; Sridhara et al., 2021; Nagaraju et al., 2023 and Sivaji and Girija, 2017). PCR analysis revealed the occurrence of *cry4Aa* in two isolates (T166 and T184), *cry4Ba* (T60), *cry11Aa* (T166) and *cyt1* (T166) in one isolate each. Nevertheless, none of the isolates showed the occurrence of *cry10Aa* gene (Table 2). Aarthi et al. (2024) reported the frequency of occurrence of *cry 10* and *cry 11* genes in two and 10 Bt strains among the 50 strains screened for their toxicity against 2nd instar *Z. cucurbitae* maggots. Salama et al. (2015) reported the occurrence of *cry 4* gene in 27.77% of the isolates.

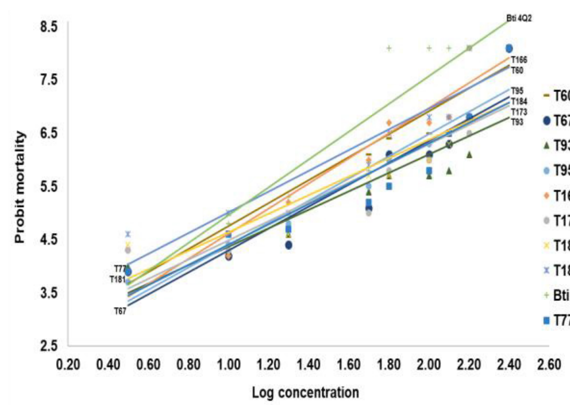

Fig. 1. Toxicity of Bt isolates to maggots

Table 1. Toxicity of Bt isolates against neonate (~12 hrs old) maggots of *Z. cucurbitae*

Isolate	Regression equation (Y = a + bx)	χ^2	LC_{50} (µg/ µl)	Confidence limits (50%)		LC_{95} (µg/ µl)	Confidence limits (95%)	
				Lower limit	Upper limit		Lower limit	Upper limit
T60	Y = 2.86 + 3.44 X	5.55	0.41	0.32	0.53	1.24	0.75	2.06
T67	Y = 6.06 + 3.61 X	4.32	0.51	0.41	0.63	1.45	0.91	2.29
T77	Y = 5.78 + 2.69 X	1.89	0.51	0.39	0.67	2.09	1.01	4.32
T93	Y = 6.15 + 4.03 X	1.48	0.52	0.42	0.63	1.33	0.88	2.01
T95	Y = 6.09 + 3.53 X	1.40	0.49	0.39	0.61	1.44	0.89	2.31
T166	Y = 3.04 + 3.36 X	10.82	0.38	0.28	0.52	1.18	0.57	2.46
T173	Y = 6.02 + 2.88 X	3.27	0.44	0.34	0.58	1.65	0.89	3.07
T181	Y = 5.92 + 3.23 X	2.58	0.52	0.41	0.66	1.67	0.96	2.94
T184	Y = 6.32 + 3.34 X	7.11	0.40	0.30	0.53	1.25	0.63	2.49
Bti 4Q2	Y = 6.43 + 3.33 X	7.98	0.37	0.27	0.51	1.15	0.50	2.66

Table 2. Characteristics of Bt isolates

Bt isolate	Colony morphology					Crystal morphology	Genes present in PCR
	Colour	Surface	Shape	Elevation	Margin		
T60	Full white	Smooth	Circular	Flat	Entire	Spherical	*cy4Ba*
T67	Full white	Smooth	Irregular	Raised	Undulate	Spherical	-
T77	Off white	Smooth	Irregular	Flat	Undulate	Cuboidal	-
T93	Off white	Smooth	Circular	Raised	Undulate	Spherical	-
T95	Off white	Fried egg	Irregular	Raised	Undulate	Bipyramidal	-
T166	Creamy white at centre encircled by off white	Smooth	Circular	Flat	Entire	Rectangular	*cry4Aa, cry11Aa* and *cyt1*
T173	Off white	Glossy	Circular	Raised	Undulate	Bipyramidal	-
T181	Off white	Irregular	Irregular	Flat	Entire	Cuboidal	-
T184	Full white	Fried egg	Circular	Flat	Undulate	Cuboidal	*cry4Aa*
Bti 4Q2	Off white	Fried egg	Irregular	Flat	Undulate	Spherical	*cry4Aa, cry4Ba, cry10Aa, cry11Aa* and *cyt1*

The occurrence of insecticidal proteins that could express toxicity to *Z. cucurbitae* maggots varied from >200kDa to ~15kDa. High toxicity in Bti 4Q2 has been reported to the combined occurrence of five genes viz., *cry4Aa, cry4Ba, cry10Aa, cry11Aa* and *cyt1* encoding for the expression of those gene-specific proteins of molecular masses ~134 kDa, ~128 kDa, ~68 kDa, ~72 kDa and ~27 kDa, respectively (Valtierra-de-Luis et al., 2020). The indigenous Bt isolates T60 and T184 expressed Cry4Ba and Cry4Aa proteins of molecular mass ~128 kDa and ~134 kDa respectively ascribing to their similar level of toxicity on *Z. cucurbitae* maggots. The maximum toxicity of T166 may be attributed to the occurrence of three genes *cry4Aa, cry11Aa* and *cyt1* expressing proteins of molecular masses ~134 kDa, ~70 kDa and ~26 kDa, respectively (Fig. 2). Cyt toxins have been reported to exhibit synergistic effects with other toxins (Valtierra-de-Luis et al., 2020). Aarthi et al. (2024) reported the combined occurrence of *cry1A, cry2A, cry10A* and *cry70* genes in all the Bt strains that displayed more than 50% mortality on the 2nd instar *Z. cucurbitae* maggots favoring the present result.

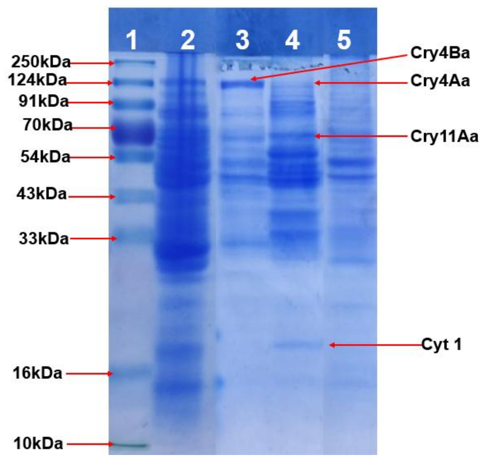

Fig. 2. Protein profiling of Bt isolates by SDS PAGE (Lane 1 – Broad range marker, Lane 2 to 5 – Bti 4Q2, T60, T166 and T184)

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AUTHOR CONTRIBUTION STATEMENT

VB designed the study. TS conducted the experiment. TS and VB wrote the manuscript. MR, GR, TE and LP provided research material, helped in conducting the experiments and reviewing and editing the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

No conflict of interest.

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