Journal of Biological Control, 28(3): 160–165, 2014

Research Article

Bio efficacy and cry profile of native *Bacillus thuringiensis* isolates against cabbage leaf webber, *Crocidolomia binotalis* and *Spodoptera litura* Fabricius

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ABSTRACT: An investigation was carried out to assess the bio-efficacy and cry profile of hundred native *Bacillus thuringiensis* isolates against Cabbage Leaf Webber *viz*., *Crocidolomia binotalis* and *Spodoptera litura* at the Department of Entomology, University of Agricultural Sciences, Dharwad. Among the hundred native isolates used, isolates DBT-763, DBT-787, DBT-754 and DBT-2370 reported 86.67 per cent mortality against *Crocidolomia binotalis* and DBT-772 showed 73.33 per cent mortality against *Spodoptera litura.* Lepidopteron specific cry genes *viz*., cry1, cry2, cry8, and cry9 genes amplified in native isolates *viz*., DBT-2367, DBT-2366, DBT-2299, DBT-2299, respectively and none of the studied native isolates shown amplification for the cry20 gene.

KEY WORDS: *Bacillus thuringiensis, Crocidolomia binotalis, Spodoptera litura,* Cry genes

(Article chronicle: Received: 11-04-2014; Revised: 21-06-2014; Accepted: 04-09-2014;)

INTRODUCTION

Cabbage (*Brassica oleracea* var. *Capitata L.*) is a commercially important cruciferous vegetable grown in winter season. It has high nutritive value besides being a good source of vitamin A, ascorbic acid, minerals and carbohydrates. Cruciferous vegetables especially cabbage and cauliflower grown in 659 thousand hectares with a production of 1.34 million tonnes per annum are economically important in India (Anonymous, 2010). However, In India, the losses of cabbage and cauliflower due to DBM is about 35 per cent with chemical control but can go upto 90 per cent in the absence of chemical control (Mohan and Gujar, 2003). The cabbage leaf webber *Crocidolomia binotalis* is another secondary pest which is emerging as primary pest and the cluster or tobacco caterpillar, *Spodoptera litura* (Fabricius) had been a sporadic pest of tobacco for many years and it has slowly emerged as an important insect pest in recent years (Gao *et al*., 2004). *Bacillus thuringiensis* (Berliner) is a gram-positive bacterium that occurs naturally in soil, water, infected insects and grain dust (Lambert and Peferoen, 1992) and is the most successful commercial bio-control agent against insect pests (Federici, 1999). *B. thuringiensis* has many advantages as bio-control agent viz., natural biocide, eco-friendly, specific to certain order of insect pests, not harmful to predators and other beneficial insects, mammals and birds. The production of proteinaceous crystalline inclusions (crystals) during sporulation are responsible for its toxicity towards a variety of invertebrates, especially insects (Sauka *et al*., 2010). The cry proteins encoded by specific cry genes are classified according to their amino acid similarity in 59 major groups divided into different classes and subclasses (*B. thuringiensis* toxin nomenclature website at http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/) (Crickmore *et al*., 1998). Any strain of *B. thuringiensis* may have more than one cry gene for toxic proteins. Recent advances in molecular biology have allowed the development of PCR based methods to detect the presence or absence of specific genes. The present study was aimed to assess the toxicity of native *B. thuringiensis* isolates against *C. binotalis* and *S. litura* and identify the presence of chosen cry genes.

MATERIALS AND METHODS

Mass multiplication of test insects Larvae of cabbage leaf webber, *Crocidolomia binotalis* (Zeller) (Lepidoptera: Pyralidae) collected from the infested fields of cabbage were reared separately on cabbage leaves in green house under insecticidal free condition (insectary). Pupae obtained from rearing of larvae were kept in a sterilized petriplate and placed in the cage of 25 cm^3 for adult emergence. When the moths started emerging, 25-30 days old small cabbage heads were provided for oviposition and moths laid eggs on

both ventral and dorsal surface of leaves. Leaves with eggs were transferred to plastic tubs of size 45 x 30 x 15 cm for mass rearing by providing ten per cent honey solution as food for adults in sterilized vial with cotton plug (Plate 1). Larvae of cluster caterpillar, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) collected from the fields were reared on cabbage leaves under insecticide free condition in the laboratory. The pupae obtained from these were kept in Petri plate and placed in a cage of 25 cm^3 for adult emergence. When moths started emerging, fresh cabbage leaves were provided for oviposition. The collected leaves were kept in a plastic basin of size 45 x 30 x 15 cm for larval emergence by providing ten per cent honey solution containing multivitamin powder for the adults as food through cotton swab kept in a sterilized glass vial. When the leaves were completely consumed by the emerged neonate larvae, the quantity of leaves was increased to allow larval development. Five day old larvae from F_1 generation were used for bioassay studies. One hundred native *B. thuringiensis* isolates with different crystal morphology collected from different regions of Western Ghats and maintained at Department of Biotechnology, UAS, Dharwad were subjected to bioassay along with HD1 to ascertain their insecticidal activity. Overnight growth of *B. thuringiensis* on Luria agar was picked and inoculated in 1 ml Luria broth (LB) and kept for growth under shaking condition at 28°C and incubated for 24 h. Then, the culture was reinoculated in Modified Glucose Media (MGM) (Aronson *et al*., 1971) and kept for 72 h at 30°C on a shaker at 200 rpm. Later the culture was serially diluted at 9:1 ratio and counts duly recorded. The concentration of *B. thuringiensis* ($1.2x10^6$ cfu/ml) was used to check for its toxicity against the test insects. Insecticidal activity of native *B. thuringiensis* isolates against test insects by using the Leaf dip bioassay described by Tabashnik and Crushing (1987). Leaf discs of 6 cm diameter were cut covering either side of midrib from untreated cabbage leaves. These discs were first dipped in nonionic surfactant (Triton X-100) followed by dipping in aqueous solution of the test isolates for about 30 seconds. Excess fluid was drained off and discs were dried under shade for 10 min before transferring to plastic containers (10 cm height and 6 cm diameter) over a moistened filter paper. Leaf discs were placed slantingly so that larvae can move and feed on either side. The bioassay was done with three replications per treatment and ten larvae of test insects were released on each disc and the container was covered with muslin cloth using a rubber band. HD1 served as standard check. Leaf disc dipped in distilled water alone served as control. The mortality was observed at 24 h, 48 h, 72 h and 96 h after treatment and data were subjected to analysis of variance (CRD) after suitable transformation (arc sine) and the means were separated by Duncan's Multiple Range Test (DMRT) (Duncan, 1955). Cry profile of the native *Bacillus thuringiensis* isolates Isolation of total DNA from the B. thuringiesis isolates Total DNA was isolated from *B. thuringiensis* isolates by following the protocol as outlined by Sambrook and Russel (2001). Specific PCR amplification of cry genes After amplifications, 10 µl of the amplicon from each tube along with 6X loading dye were loaded on 0.8 to 1.2 per cent agarose gel in 1X TAE of pH8.0 DNA EcoRI/ Hind III digest or -DNA Hind III digest or 100bp DNA ladder was used as DNA molecular weight marker.

Table. 1 Details of the lepidopteron specific degenerate primers

Sl. no	Gene	Sequences	Size (bp)	References
1	*cry*1	FP:AGGCGGTGAATGMBCTGTTTAC RP:CGTTTATCHGCCGCRTGAATC	940	Johnson, 2011
2	*cry*2	FP:GTTATTCTTAATGCAGATGAATGGG RP:CGGATAAAATAATCTGGGAAATAGT	689-701	Ben dov *et al*., 1997
3	*cry*8	FP:GATACRGAAACRTATCCAACGT RP:CATATCTWTRRTTCGGTTGRACTGTA	900	Johnson, 2011
4	*cry*9	FP: GGTTCTCAAAGATCCGTGTA RP: MDATYTCTAKRTCTTGACTA	1050	Juarez Perez *et al*., 1997
5	*cry*20	FP:CAATCCCTGGCTTCACTCGT RP:CCGCGGGCATTAGGATT	490	Ejiofar and Johnson, 2002

The choice of the marker and percentage of agarose gel were based on the expected size of the amplicon. The electrophoresis was done at 90V for 1 h. After separation of the amplicons, gels were visualized under UV light and documented by using a gel documentation system (G BOX from Syngene Cambridge, UK). For amplification of cry genes from native *Bacillus thuringiensis* isolates The total DNA obtained was diluted to 125 ng and used as template DNA,

Taq DNA Polymerase (3U/µl) was obtained from, 10x Taq assay buffer and Mgcl2 were obtained from M/s Merck Millipore, dNTP's individual dNTP's such as dATP, dGTP, dCTP and dTTP were obtained from Eppendorf, Germany. Five sets of standard primers were used in the present study (Table 1), PCR amplifications were performed in the automated thermal cycler (Eppendorf 5331) with an initial denaturation [94°C, 3 sec] followed by 39 cycles of denaturation [92°C, 30 sec], annealing [cry1, cry2, cry8, cry9 and cry20 genes amplified with annealing temperatures of 45.0, 51.6, 51.0, 45.0 and 42.7 respectively for 1 min], and extension [72°C, 2 min] with a single final extension [75°C, 20 min], kept on hold at 4°C and the samples were stored at 4°C in refrigerator until further use. PCR reaction mix required was prepared by adding 3.12 µl of sterile distilled water, 1 µl of taq assay buffer, 1.5 µl of dNTP's (1mM), 3 µl of forward, 3 µl of reverse primers, 1.5 µl of Mgcl2 (25mM) and 1 µl of template DNA (125 ng) was added from the respective samples to make up 15 µl.

RESULTS AND DISCUSSION

Data of different native *B. thuringiensis* isolates against cabbage leaf webber, *C. binotalis* is presented in Table 2. Among the hundred different native *B. thuringiensis* isolates evaluated for bioassay, the mortality of cabbage leaf webber increased over the period of time and ranged from 0 to 86.67 per cent. The isolate DBT-763 recorded highest mortality of 86.67 per cent with quicker knock down effect over HD1. Isolates viz., DBT-754, DBT-2370 and DBT-787 recorded 86.67 per cent mortality and were statistically on par with DBT-763 at 96 HAT. Rest of the isolates registered mortality of 70.00 per cent and less mortality after 96 HAT. The results are in concordance with the findings of Yadav (2007) who reported Tamil Nadu isolates (Tx-201 and Tx-202) caused 100 per cent mortality which were superior to HD1 and Nethravathi *et al*., (2009) noticed that the cabbage leaf webber was susceptible to 2422/C from Chikkamagalur to an extent of 80 per cent after 96 HAT. Efficacy of different native *B. thuringiensis* isolates against cluster caterpillars, *Spodoptera litura*, is presented in Table 3. The larval mortality of *S. litura* ranged from 0 to 73.33 per cent in one hundred isolates after 96 HAT. The highest mortality was recorded in DBT-772 with 73.33 per cent followed by HD1 and DBT-764 with 66.67 per cent mortality. DBT-754 and DBT-787 recorded 63.33 per cent mortality and DBT-2372 and DBT-388 recorded 60.00 per cent mortality. Similar findings reported by Manimegalai *et al*., (2005), Marutesh (2007), and Yadav (2007). Polymerase chain reaction (PCR) was employed, to identify the presence of different cry genes using the cry specific primers. Five sets of Lepidopteron specific primers were used in PCR amplification. Among reference strains HD1 amplified for cry1, 4D4 amplified for cry2, 4AT1 amplified for cry8 and cry9 and 4AP1 amplified for cry20 (Plate 3). Based on the bioassay results only eleven isolates showing mortality of more than 75.00 per cent [except DBT-2372 because of its high efficacy against other two insects] were diagnosed for presence of Lepidopteron specific cry genes. Cry1 gene was observed in DBT-763, DBT-764, DBT-754, DBT-772, DBT-2369 and DBT-2299 isolates, cry2 gene was observed in DBT-764, DBT-2368, DBT-772 and DBT-2370 isolates, cry8 was detected in two isolates *viz*., DBT-787 and DBT-2368, cry9 was amplified in two isolates *viz*.,DBT-388 and DBT-2370 and cry20 was not amplified in any of the eleven isolates diagnosed (Plate 4). Bravo *et al*. (1998) found that crystal protein belonging to the cry1 and cry9 groups were toxic for lepidopteran insect. The cry3, cry7, cry8 proteins are active against coleopteran insects. The cry5, cry12, cry13 and cry14 proteins are nematicidal. The cry11, cry21 and cyt proteins are toxic to dipteran insects.

Similarly, classification of cry genes based on amino acid similarity for over 200 cry genes have been categorized into 47 classes and sub classes (Crick more *et al*., 2002). Among the isolates studied, cry1 occurred in the highest frequency of (54.5%), followed by cry2 (36.36%), cry8 and cry9 (18.18%). The variations in efficacy against different Lepidopteron may be due to varying number of cry genes and the absence of specific binding sites as shown by other workers Knowles (1994) and Yadav (2007).

ACKNOWLEDGEMENT

We thank the management of DBT, New Delhi and Department of Biotechnology for their financial support.

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Table. 2 Efficacy of different native *Bacillus thuringiensis* isolates against cabbage leaf webber, *Crocidolomia binotalis* Z. at different intervals

Sl. No.	Isolates	Mean per cent mortality at different intervals after treatment			
		24 h	48 h	72 h	96 h
1	DBT-2288	2.50 (9.09)h	10.00 (18.43)op	36.67 (37.21)h	53.33 (46.90)h
2	DBT-2299	7.50 (15.32)f	30.00 (32.99)hij	56.67 (48.83)f	63.33 (52.75)fg
3	DBT-2304	5.00 (12.21)g	7.50 (15.32)pqr	16.67 (23.85)pqr	50.00(44.90)hij
4	DBT-2366	2.50 (9.09)h	30.00 (32.70)hij	56.67 (48.83)f	60.00 (50.83)g
5	DBT-2367	2.50 (9.09)h	33.33 (35.00)gh	46.67 (43.06)g	63.33 (52.75)fg
6	DBT-2367	5.00 (12.21)g	36.67 (37.21)f	53.33 (46.90)f	76.67 (61.20)de
7	DBT-2369	2.50 (9.09)h	20.00 (26.55)lm	46.67 (43.06)g	80.00 (63.41)c
8	DBT-2370	23.33 (28.77)d	43.33 (41.14)d	53.33 (46.90)f g	86.67 (68.83)b
9	DBT-2371	2.50 (9.09)h	2.50 (9.09)s	46.67 (43.06)g	63.33 (52.75)fg
10	DBT-2372	2.50 (9.09)h	50.00 (44.98)c	63.33 (52.75)e	66.67 (54.76)e
11	DBT-2384	2.50 (9.09)h	30.00 (33.20)hi	56.67 (48.83)f	63.33 (52.75)fg
12	DBT-763	30.00 (32.99)c	53.33 (46.90)cd	83.33 (66.61)a	86.67 (72.76)a
13	DBT-764	14.17 (20.24)e	36.67 (37.21)f	66.67 (54.76)d	80.00 (63.41)d
14	DBT-3099	2.50 (9.09)h	5.00 (12.21)rs	20.00(26.55)nopq	66.67 (54.76)e
15	DBT-3075	2.50 (9.09)h	30.00 (33.20)hi	33.33 (35.20)hi	73.33 (58.98)d
16	DBT-3098	5.00 (12.21)g	33.33 (35.00)gh	43.33 (41.05)g	53.33 (46.90)h
17	DBT-772	26.67 (30.98)cd	33.33 (35.20)gh	53.33 (46.90)f g	63.33 (52.75)fg
18	DBT-787	26.67 (30.28)cd	43.33 (41.05)d	63.33 (52.75)e	86.67 (72.26)bc
19	DBT-754	8.33 (14.91)fg	40.00 (39.05)e	63.33 (52.75)e	86.67 (72.26)bc
20	DBT-388	40.00 (39.22)ab	56.67 (48.83)b	73.33 (58.98)c	76.67 (61.20)de
21	HD1	43.33 (41.14)a	63.33 (52.75)a	80.00 (63.41)b	86.67 (68.83)c
22	Control	2.50 (9.09)h	2.50 (9.09)s	5.00 (12.21)vw	5.00 (12.21)y
	CV (%)	4.11	3.89	3.95	1.54
	SEm ±	0.65	0.43	0.60	0.33
	CD at 1%	2.39	1.59	2.24	1.22

* Values within parentheses indicate the arcsine transformed values.
The values superscripted by same alphabet are statistically on par with each other by DMRT.

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Table. 3 Efficacy of different native *Bacillus thuringiensis* isolates against *Spodoptera litura* (Fab.) at different intervals

Sl. No.	Isolates	Mean per cent mortality at different intervals after treatment			
		24 h	48 h	72 h	96 h
1	DBT-2265	2.50 (9.09)b	5.00 (12.21)fg	26.67 (30.77)ef	56.67 (48.91)de
2	DBT-2369	2.50 (9.09)b	2.50 (9.09)g	13.33(21.14)jkl	56.67 (48.83)de
3	DBT-764	5.00 (12.21)a	5.00 (12.21)fg	26.67 (30.98)ef	66.67 (54.97)bc
4	DBT-3099	2.50 (9.09)b	5.00 (12.21)fg	26.67 (30.98)ef	53.33 (46.90)e
5	DBT-3075	2.50 (9.09)b	2.50 (9.09)g	16.67(23.85)hij	53.33 (46.90)e
6	DBT-3098	2.50 (9.09)b	2.50 (9.09)g	23.33 (28.27)fg	56.67 (48.91)de
7	DBT-772	2.50 (9.09)b	14.17(20.73)d	43.33 (41.05)b	73.33 (58.98)b
8	DBT-787	2.50 (9.09)b	2.50 (9.09)g	33.33 (35.20)cd	63.33 (52.75)bc
9	DBT-754	2.50 (9.09)b	5.00 (12.21)fg	23.33 (28.77)fg	63.33 (52.84)bc
10	DBT-388	2.50 (9.09)b	5.00 (12.21)fg	30.00 (32.70)de	60.00 (51.12)cd
11	HD1	5.00 (12.21)a	6.67 (12.29)e	23.33(28.27) fg	66.67(54.76) bc
12	Control	2.50 (9.09)b	2.50 (9.09)g	2.50 (9.09)o	2.50 (9.09)o
	CV (%)	4.03	4.16	4.39	3.38
	SEm ±	0.30	0.52	0.53	0.93
	CD at 1%	1.13	1.93	1.96	3.43

* Values within parentheses indicate the arcsine transformed values.
The values superscribed by same alphabet are statistically on par with each other by DMRT

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