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Biocidal Activity of *Bacillus thuringiensis* on *Plutella xylostella* L.

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Fifty native *Bacillus thuringiensis* isolates of Dharwad were evaluated for their insecticidal activity against third instar larvae of *Plutella xylostella*. Toxicity of these isolates ranged from nill to 92.00 per cent. Most promising ones are DBT-388 (92.00%) and DBT-772 (86%) comparable with reference strain HD1 (84%). Crude protein extraction was done from DBT-388 and DBT-772 isolates along with HD-1. LC_{50} values were 1738, 2113 and 2427 ppm for DBT-388, DBT-772 and HD-1 respectively. Spore crystal complex count also taken from DBT-388 and HD-1 to calculate the International Unit (IU) /ml and to to find out its potency. DBT-388 and HD-1 had a count of 42,000 and 32,000 IU/ml, respectively when measured on *P. xylostella*. DBT-388 produced more spores than the HD-1. Effective isolates (mortality greater than 75%) were screened to know the spectrum of *cry*1, *cry*2, *cry*8, *cry*9 and *cry*20 genes present in them. *cry*1 gene was amplified in eleven isolates, *cry*2 in eight isolates whereas *cry*8 and *cry*9 in three and six isolates, respectively. *cry*20 was not amplified in any of the twelve isolates.

Key words: *Bacillus thuringiensis*, *Plutella xylostella*, toxicity, LC_{50} values and *cry* genes.

This is a ubiquitous gram-positive, rod-shaped and sporulating bacterium that has been isolated worldwide from a great diversity of ecosystems (Hofte and Whiteley,1989; Raymond *et al.*, 2010). This bacterium produces several types of toxins : alpha-exotoxin ("heat-labile exotoxin," possibly lecithinase C), beta-exotoxin ("heat-stable exotoxin," an adenine-containing compound), delta endotoxin (crystal protein) and the "louse factor" (Whiteley and Schnepf, 1986). Its principal characteristic during sporulation is the synthesis of a crystalline inclusions containing proteins known as δ-endotoxins which are well known for their insecticidal properties (Aronson, 1993; Frankenhuyzen, 2009). These toxins have been successfully used as bioinsecticides against caterpillars, beetles, and flies, including mosquitoes and blackflies. This has led these products to become the best selling biological insecticides and majority of microbial pesticides are made from subspecies and strains of *B. thuringiensis* (Sanchis and Bourguet, 2008). Crystal solubilization is dependent on gut pH. For the majority of the crystal inclusions produced by *Bt*, gut conditions must be strongly alkaline in order to achieve dissolution (Hofmann *et al.*, 1988). Gut pH is, therefore, one of the factors that help to determine potential toxicity. In lepidoptera, specificity was due to the extensively alkaline midgut environment that is required to solubilize the prototoxin into the active form (Knowles and Dow, 1993).

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Throughout the world diamondback moth *Plutella xylostella* L. is considered as the main insect pest of cruciferous vegetables and oilseed crops like canola and mustard (Furlong *et al.*, 2013). The estimated worldwide cost for controlling *P. xylostella* is US $45 billion annually (Zalucki *et al.*, 2012). Though it is one of the most studied insect pests in the world, yet it is among the the most difficult pests to control. The management of this pest to ensure the stable and high output of crops is a great challenge in agricultural field and therefore, insecticide use is most widely practiced for control. But heavy use of insecticide is causing residual problem as well as development of pesticide resistance in the pests. There is a need for developing safe and eco-friendly alternatives to chemical insecticides which are effective, bio-degradable and do not leave any harmful effect on environment.

In this context the present study aims at exploring the insecticidal activity against *P. xylostella* from some *B.thuringiensis* isolates, which are safe to non-targeted organisms, humans and the environment; and also to characterize the *cry* genes present in the isolates.

MATERIALS AND METHODS

Mass multiplication of *Plutella xylostella*

Diamondback moth (DBM) was mass cultured in the laboratory at approximately 12:12 (L:D) photoperiod and 27±2 ^{0}C temperature following the method given by Liu and Sun (1984) with some modifications. Larvae were reared on cabbage leaves collected from the plants raised under insecticide free conditions. Ten per cent honey solution containing multivitamin powder was provided for the adults. Third instar larvae (0.5 ± 0.15 cm; 1.65 ± 0.20 mg) were used for the bioassay (Vastrad, 2000).

Preparation of *B. thuringiensis* culture for bioassay

Fifty isolates were screened against *P. xylostella*. Single colony forming unit from the fourway streaked growth was inoculated in 1 ml Luria broth (LB) in an eppendorf tube. This was kept for growth under shaking condition at 37 ^{0}C and incubated overnight. Then the culture was reinoculated in modified 'G' medium (MGM) broth (Aronson *et al.*, 1971) and kept for 72 hr at 37 ^{0}C on a shaker at 180 rpm. Later the culture was serially diluted at 9:1 ratio and plated on LA media by spread plate technique and kept for growth at 37 ^{0}C overnight for taking colony count before arriving at the concentration of *Bt* (1.2×10^6 cfu/ml) to assess it's toxicity against the test insects (Shilpa, 2005). Leaf dip bioassay method was followed as described by Tabashnik and Cushing (1987). HD-1 served as standard check and leaf disc dipped in distilled water alone served as control. Later per cent larval mortality was observed at 24, 48 and 72 hr after treatment and data were subjected to analysis of variance with suitable transformation (arc sine) and the means were separated by Duncan's Multiple Range Test (Duncan, 1955). Later crude protein extraction was done from the promising isolates along with HD-1 by following the method suggested by Dulmage *et al.* (1971) and tested at five different concentrations (800, 1000, 2000, 3000, 4000 and 5000 ppm). Bioassay results were subjected to statistical analysis with SPSS software for the dose mortality response (LC_{50} and LC_{99}) using probit analysis. Spore crystal complex count was also taken to assess their potency and to calculate the International Unit (IU) /ml.

Total DNA was isolated from potent *B. thuringiensis* isolates by following the protocol given by Sambrook and Russel (2001). To investigate the presence of lepidopteran specific *cry*1, *cry*2, *cry*8, *cry*9 and *cry*20 genes, polymerase chain reaction was employed. Annealing temperatures set for *cry*1, *cry*2, *cry*8, *cry*9 and *cry*20 genes are 57.5, 51.6, 51.0, 45.0 and 45.0º C respectively.

Confirmation of DBT-388 and DBT-772 strains up to species level was done by 16S Rrna based sequencing. PCR amplified product of 16S rRNA genes fragments were sequenced at Xceliris, Gujarat. The sequences were compared with the available sequences in NCBI database using BLASTn algorithm. The identification was based upon pair wise alignment and phylogenetic study with the already existing sequences in database.

RESULTS

Among the fifty isolates, mean per cent larval mortality ranged from 0.00 to 92.00 per cent at the end of 72 hr of treatment (Table 1). Highest mortality was observed in DBT-388 (92.00%)

Table.1 Bioefficacy of native isolates of *Bacillus thuringiensis* on *Plutella xylostella*

Sl. No.	Isolates	Mean per cent corrected mortality at different intervals after treatment		
		24 hr	48 hr	72 hr
1	DBT – 182	0 (5.74)e	14 (22.52)d-g	30 (33.7)e-i
2	DBT – 186	0 (5.74)e	12 (20.94)d-h	32 (35.02)e-h
3	DBT -787	20 (27.26)a	48 (44.41)ab	82 (65.81)bc
4	DBT -179	0 (5.74)e	20 (26.99)d	44 (42.06)d
5	TX- 201	12 (20.94)b	38 (38.56)a-c	78 (63.11)bc
6	DBT – 177	0 (5.74)e	10 (19.36)e-i	36 (37.41)b-g
7	DBT -169	0 (5.74)e	8 (16.64)g-j	28 (32.51)f-j
8	DBT -107	0 (5.74)e	14 (22.52)d-g	38 (38.6)d-f
9	DBT -499	0 (5.74)e	6 (13.91)h-k	26 (31.2)g-k
10	DBT -502	0 (5.74)e	16 (24.1)d-f	38 (38.56)d-f
11	DBT -388	8 (16.64)c	50 (45.56)a	92 (76)a
12	DBT -2370	0 (5.74)e	4 (11.19)j-l	12 (19.8)o-q
13	DBT -3893	0 (5.74)e	4 (11.19)j-l	14 (22.52)m-p
14	DBT -772	10 (19.36)bc	44 (42.1)a-c	86 (69.16)b
15	DBT -106	0 (5.74)e	0 (5.74)l	10 (19.36)o-q
16	DBT -109	0 (5.74)e	10 (17.07)f-j	20 (26.73)i-n
17	DBT -110	0 (5.74)e	0 (5.74)l	10 (19.36)o-q
18	DBT -112	0 (5.74)e	4 (11.19)j-l	8 (16.64)pq
19	DBT -123	0 (5.74)e	8 (16.64)g-j	18 (25.68)j-o
20	DBT -124	0 (5.74)e	0 (5.74)l	10 (19.36)o-q
21	DBT – 192	0 (5.74)e	8 (16.64)g-j	24 (29.89)h-l
22	DBT -195	0 (5.74)e	16 (24.1)d-f	30 (33.82)e-h
23	DBT -172	0 (5.74)e	0 (5.74)l	10 (19.36)o-q
24	DBT -104	0 (5.74)e	10 (18.22)f-j	26 (31.2)g-k
25	DBT -2510	4 (11.19)d	38 (38.6)a-c	80 (64.46)bc
26	DBT -2336	0 (5.74)e	34 (36.21)c	80 (64.46)bc
27	DBT -3008	10 (19.36)bc	46 (43.25)a-c	82 (65.81)bc
28	DBT -3009	4 (11.19)d	40 (39.8)a-c	82 (65.81)bc
29	DBT -2500	20 (27.26)a	46 (43.25)a-c	80 (64.79)bc
30	DBT -113	2 (8.46)de	6 (12.77)i-l	14 (21.11)n-q
31	DBT -116	4 (11.19)d	10 (17.07)f-j	20 (25.2)k-o
32	DBT -185	2 (8.46)de	10 (18.22)f-j	28 (32.51)f-j
33	DBT -197	2 (8.46)de	12 (20.94)d-h	30 (33.7)e-i
34	DBT -125	4 (11.19)d	14 (22.52)d-g	24 (29.89)h-l
35	DBT- 754	10 (19.36)bc	42 (40.95)a-c	80 (64.46)bc
36	AIM 213 (a)	8 (16.64)c	38 (38.6)a-c	78 (62.78)bc
37	DBT- 167	0 (5.74)e	4 (11.19)j-l	16 (24.1)l-o
38	DBT- 190	0 (5.74)e	18 (25.68)de	40 (39.8)de
39	DBT- 2299	0 (5.74)e	34 (36.21)c	80 (64.46)bc
40	DBT- 173	0 (5.74)e	10 (19.36)e-i	26 (31.2)g-k
41	DBT- 125	0 (5.74)e	2 (8.46)kl	10 (19.36)o-q
42	DBT- 100	0 (5.74)e	2 (8.46)kl	8 (15.49)q
43	DBT- 184	0 (5.74)e	10 (19.36)e-i	22 (28.31)h-m
44	DBT- 2712	4 (11.19)d	38 (38.6)a-c	80 (64.46)bc
45	DBT- 102	0 (5.74)e	4 (11.19)j-l	8 (15.49)q
46	DBT- 204	0 (5.74)e	0 (5.74)l	0 (5.74)r
47	DBT- 198	0 (5.74)e	4 (11.19)j-l	22 (28.57)h-m
48	DBT- 178	0 (5.74)e	10 (19.36)e-i	32 (35.02)e-h
49	TX- 29	8 (16.64)c	36 (37.41)bc	74 (60.22)c
50	HD – 1	0 (5.74)e	46 (43.25)a-c	84 (67.48)b
51	Control	0 (5.74)e	0 (5.74)l	0 (5.74)r
S.Em±	1.34	2.174	2.1	
C.D	3.733	6.05	5.84	

Values in parentheses are Arcsine transformed values, Means followed by same letters in a column do not differ significantly (0.05) by DMRT

followed by 86.00 per cent in DBT-772. DBT-2336, TX-29, TX-201, AIM 213 (a), DBT-2510, DBT-2500, DBT-3009, DBT-3008, DBT-754, DBT-787, HD-1 and DBT- 3008 reduced larval load in between 78.00 to 82.00 per cent which were statistically on par which each other. As the highest mortality is observed from DBT-388 and DBT-772 further crude protein was extracted from these and tested to arrive at LC_{50} values. Crude protein powder of DBT-388, DBT-772 and HD-1 caused 100, 96 and 92 per cent mortality, respectively at 5000 ppm after 72 hr of treatment period. LC_{50} values calculated from crude protein powder for these isolates were 1738, 2113 and 2427 ppm for DBT-388, DBT-772 and HD-1, respectively (Table 2). Spore crystal complex count was taken from best isolate of all fifty i.e., DBT-388 along with HD-1 and they were compared. Count revealed that DBT-388 produced more spores than the HD-1. DBT-388 and HD-1 had a potency of 42,000 and 32,000 IU/ml, respectively when measured on *P. xylostella*.

*cry*1 was amplified in DBT- 2510, DBT-772, DBT- 3009, DBT- 2336, HD-1, DBT- 2712, DBT-2500, DBT- 2299, DBT- 754, DBT- 3008 and DBT-787. *cry*1 was amplified in all isolates except DBT-388. *cry* gene profile of the *B. thuringiensis* isolates is given in table 3. *cry*2 was observed in DBT- 772, DBT- 3009, DBT- 2336, HD-1, DBT- 388, DBT- 2299, DBT- 754 and DBT- 3008 isolates. *cry*8 was amplified in three isolates DBT- 772, DBT- 2299 and DBT-754. *cry*9 was seen in six isolates DBT- 2510, DBT-3009, DBT- 2336, DBT- 388, DBT- 2299 and DBT-3008 (Table 4). *cry*20 was not amplified in any of the isolates. *cry*1 gene was present in highest frequency (0.91) followed by *cry*2 (0.66) *cry*9 (0.5) and *cry*8 (0.25). BLAST analysis reconfirmed DBT-388 and DBT-772 as *Bacillus thuringiensis* with 99 and 96 per cent homology, respectively. Total DNA and *cry* profiles of native *B. thuringiensis* isolates is shown in plate 1 and 2.

Table 2. Dose mortality response of *Plutella xylostella* to the crude protein powder of DBT-388, DBT-772 and HD-1 *Bacillus thuringiensis* isolates

Sl. No.	Isolates	LC_{50} conc. (ppm)	95% Confidential limits		LC_{99} conc. (ppm)
			Lower limit (ppm)	Upper limit (ppm)	
1	DBT-388	1738.39	1126.93	2447.34	6401.53
2	DBT- 772	2113.61	1878.88	2369.44	6648.45
3	HD-1	2427.60	2162.28	2727.22	7407.71

Table 3. *cry* gene profile of the *B. thuringiensis* isolates

Isolate Number	*cry* 1	*cry* 2	*cry* 8	*cry* 9	*cry* 20
DBT- 2510	+	-	-	+	-
DBT- 772	+	+	+	-	-
DBT- 3009	+	+	-	+	-
DBT- 2336	+	+	-	+	-
HD-1	+	+	-	-	-
DBT- 2712	+	-	-	-	-
DBT- 2500	+	-	-	-	-
DBT- 388	-	+	-	+	-
DBT- 2299	+	+	+	+	-
DBT- 754	+	+	+	-	-
DBT- 3008	+	+	-	+	-
DBT- 787	+	-	-	-	-
S-Standard	+	+	+	+	+
Standard used	HD1	4 D4	4 AP1	4 AP1	4 AT1

+ Presence of crystal gene; - Absence of crystal gene

Table 4. *cry* gene profile in individual native isolates of *B. thuringiensis*

Sl. No	Isolate Number	*cry* gene content
1.	DBT- 2510	*cry*1, *cry*9
2.	DBT- 772	*cry*1, *cry*2, *cry*8
3.	DBT- 3009	*cry*1, *cry*2, *cry*9
4.	DBT- 2336	*cry*1, *cry*2, *cry*9
5.	HD-1	*cry*1, *cry*2
6.	DBT- 2712	*cry*1
7.	DBT- 2500	*cry*1
8.	DBT- 388	*cry*2, *cry*9
9.	DBT- 2299	*cry*1, *cry*2, *cry*9, *cry*8
10.	DBT- 754	*cry*1, *cry*2, *cry*8
11.	DBT- 3008	*cry*1, *cry*2, *cry*9,
12.	DBT- 787	*Cry*1

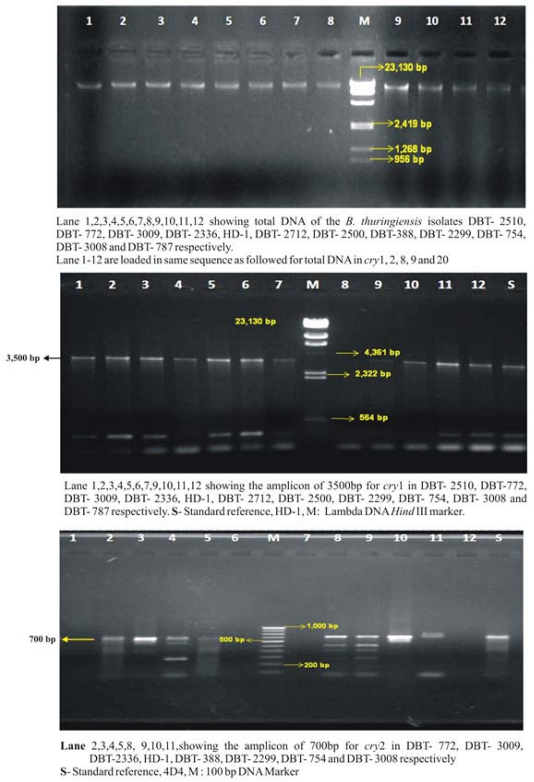

Plate 1. Total DNA, *cry*1 and *cry*2 profiles of native *B. thuringiensis* isolates

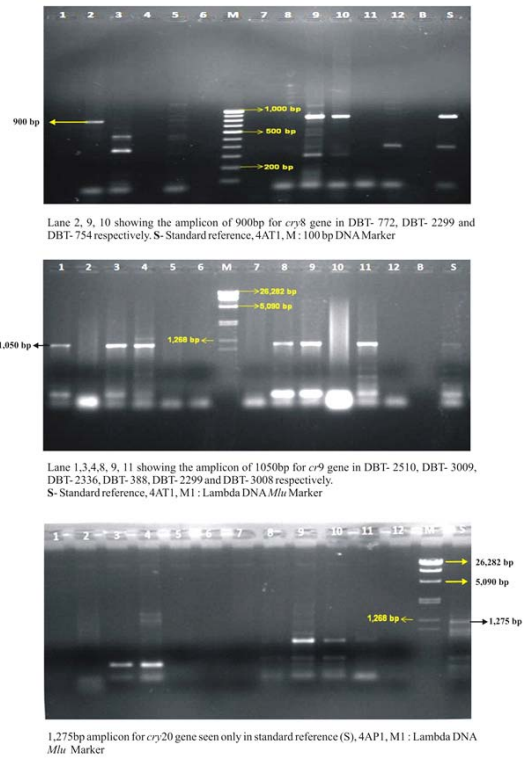

Plate 2. *cry*8, *cry*9 and *cry*20 profiles of native *B. thuringiensis* isolates

DISCUSSION

Among fifty isolates tested against *Plutella xylostella*, mean per cent larval mortality ranged from nill to 92 per cent. Four isolates reported mortality less than 10.00 per cent, ten isolates recorded mortality ranging from 10 to 20 per cent, 18 isolates showed mortality in between 20 and 50 per cent, 13 isolates in between 50 to 80 per cent and five isolates reported above 80 per cent mortality. HD1 has registered 84 per cent.

Similar differences in effectiveness among the strains and subspecies of *B. thuringiensis* against *Plutella xylostella* and other insects have been reported by earlier workers (Kaur *et al.*, 2006, Monnerat *et al.*, 2007 and Nethravathi and Hugar, 2010). The differences in the efficacy of different isolates of *B. thuringiensis* has been might be due to the differences in the carbohydrate affinity of the domain II which results in variable binding specificity with the receptors at the brush border membrane of the insect larvae, causing difference in toxicity of the cry protein (Burton *et al.*, 1999). Higher toxicity of native *Bt* isolates than reference strain (HD1) against *P. xylostella* was reported by Shilpa (2005), Nethravathi (2009). Prabhakar (2011) reported 90.00 per cent mortality in Tx-379, 86.67 per cent mortality in AIM-72 (1), AIM-72 (2) and in reference strain HD1 recorded 83.33 per cent mortality. The present results are in agreement with above authors who have also reported differences in the efficacy among isolates.

Nethravathi (2009), reported that 1674/a isolate caused cent per cent mortality against DBM larvae with the LC_{50} value of 633.0 ppm. A cumulative mortality of 100 per cent was recorded by Geeta and Alagawadi (2012) in three *Bt* isolates *viz.*, UK-13C, UK-762D, UK-25A followed by UK-52A, UDP-420B, DK-45B and DK-6B (93.00, 86.67, 86.67 and 83.33 respectively) at 2000 ppm after 72 hr of treatment.

International units (IU/mg or ml) specify the amount of delta-endotoxins produced by the *Bt* strain. Potency is a measure of the number of

effective killing units of the product (crystal proteins, spores, *etc.*). Potency and efficacy depend on how much delta-endotoxin a formulation contains and the level of this active ingredients determines how effectively a formulation performs (Dulmage and Beegle, 1984). DBT-388 isolate had a potency of 42,000 IU/ml when tested against *P. xylostella*, where as for HD-1 it was 32,000 IU/ml. DBT-388 produce more delta endotoxins than the HD-1 and this might be the reason for better result of DBT-388. The amount of cry toxins and the relative amount of effective toxins in the spore crystal complex will decide the efficacy of the isolate. The high production of spore crystal complex could be important in the economics of commercialization (Gorashi *et al.*, 2014).

Most of the isolates had more than one *cry* genes. *cry*1 gene was the most abundant gene (91.66%), followed by *cry*2 (66.66%), *cry*9 (50%) and *cry*8 (25%). Variations in the *cry* gene contents and distribution are likely associated with the differences in the biological, geographical and ecological properties of the collection areas (Lopez *et al.*, 2009). Gislayne *et al.* (2004) found that out of 218 isolates collected from Brazil *cry*1 gene was the most abundant (48.0%). Patel *et al.* (2013) reported diversity of *cry* genes from different parts of the India. They observed that *cry* 1 was the most abundant of all the genes. It indicates that *cry*1 is the most abundant of all the genes. Comparison of the insecticidal profile for the isolates based on their *cry* content indicated that most of the isolates obtained in this study had the potential to be active against Lepidoptera. More than one gene was also noted in many of the isolates and all these strains were potent against *Plutella xylostella*. Martinez and Caballero (2002) found eight different *cry* genes in a single isolate. Mixture of toxins in these isolates might have induced appreciable amount of mortality in the test insect. Strain that produces mixture of toxins is more toxic than the strain that produces only one *cry* toxin (Mohan and Gujar, 2002). Differences in toxin expression or synergistic effects between some cry toxins, could account in toxicity of *Bt* strains (Monnerat *et al.*, 2007). So, the synergistic effect of *cry*9 and *cry*2 in DBT-388 might be the reason for its high insecticidal activity than all others strains. Though this combination of *cry*9 and *cry*2 genes is present in other isolates (DBT-3009, DBT-3008, DBT-2336 and DBT-2299,) the synergism might not have occurred due to the presence of other additional genes and the per cent mortality recorded was 82 (DBT-3009), 82 (DBT-3008), 80 (DBT-2336) and 80 (DBT-2299) per cent respectively which is lesser than DBT-388.

CONCLUSION

There is widespread concern over negative impact of insecticides on environmental and human health. The present study has highlighted the effective isolates like DBT-388 and DBT-772 which could be starting point for developing them as bioinsecticides, which are effective, bio-degradable and do not leave any harmful effect on environment. Further studies are needed on mass filtration units (*Bacillus thuringiensis*) and formulation of these potent isolates along with the field evaluation in various agro-ecosystems.

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