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Insecticidal activity of the isolated and characterized native *Bacillus thuringiensis* (Berliner) against *Plutella xylostella* (Linnaeus)

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Abstract: Twenty two isolates of *Bacillus thuringiensis* (Berliner) (Bt) were, characterized from eleven different cropping ecosystems and evaluated for the pathogenic activity against *Plutella xylostella* (L). Based on the production of parasporal crystal eighteen isolates from present study and four previously isolated isolates were identified as Bt after examining the 212 Bt like colonies. Microscopic observations of crystal staining exhibited diversity as eight isolates were having irregular shape, six of spherical, four both spherical and cuboidal, three with only cuboidal and one with bipyramydal shaped crystals. All the isolates that were found to be positive to V-P reaction, nitrate reduction, catalase production, casein hydrolysis, oxidase test and carbohydrate fermentation like, glucose, sucrose, trehalose test and negative for arginine hydrolysis and esterase activity. The isolates MDS-2, KMS-2, KMF, TNP, were found to be negative for starch hydrolysis while MDC, TNP, GTP for citrate utilization. Bt tested for their insecticidal activity against *P. xylostella* registered between 13.33 to 90.00 per cent mortality at 72 hr after feeding. Reference strain HD1 showed highest mortality of 100 per cent. While GBP-2, BGC-1, BGC-2, BGM-2 and KMP exhibited more than 80 per cent mortality.

Key words: *Bacillus thuringiensis,* Bioassay, Parasporal crystal, *Plutella xylostella*

Introduction

Bacillus thuringiensis (Berliner) (Bt) is ubiquitous, gram positive, soil bacterium and well known biocontrol agent. The prevalence of this species is not restricted and has been isolated worldwide from many habitats. The insecticidal property of Bt is due to crystalline inclusions, consist of one or more polypeptides called insecticidal crystal protein (ICP) or delta endotoxins (ä-endotoxin) produced during sporulation (Nagamatsu *et al.,* 1998). The protoxins are digested by enzymes in the insect midgut to form delta endotoxins, which bind to the midgut cells of insects, cause cell lysis through ion pore formation in the midgut leading to cessation of feeding and death of insects. Because of its insecticidal activity, Bt has been used for a long time as a biopesticide. However, it is still necessary to search for more toxins to control insect, particularly developing resistance against such pesticides, and also to provide alternatives for chemical insecticides.

Among the lepidopteran insect pests of cultivated plants, Diamondback moth (*P. xylostella*) an oligophagous pest feeding only on the plants of family Brassicaceae is one of the most widely distributed insects in the world. This insect pest can lead an up to 52 per cent loss of the market yield of cabbage (Lin *et al.,* 2013). To combat the menace of this pest, integrated pest management strategies have been developed with little success. This is mainly because the pest has developed resistant not only to chemical insecticides but also to the Bt having certain individual toxins. This necessitates the search for Bt strains with novel toxins for management of insect pests.

Meager work on isolation and characterization of Bt isolates was made on Bt particularly in northern Karnataka experiencing high temperature, dry weather, less rainfall and saline soil. The genetic diversity of Bt isolates differs from region to region and also between the cropping ecosystems. Isolation of Bt from such adverse climatic condition and different cropping ecosystem would results in isolation of novel temperature tolerant as well as salt and dry weather tolerant strains. Bt investigation was carried out on various aspects like isolation of novel strains of Bt from different cropping ecosystems, morphological, biochemical characterization, evaluation of native isolates against *P. xylostella.*

Material and methods

The study was aimed to explore the local isolates of Bt from the soils of different cropping ecosystem, their morphological, biochemical characterization and evaluation against Diamond back moth. Total of eighteen soil samples collected were subjected to isolation by following sodium acetate selective process method. Selected colonies were purified by repeated streaking on T3 medium and then stored at 4°C for further studies. Total eighteen isolates collected from different cropping ecosystem and four previously isolated Bt isolates collected from paddy ecosystem of upper Krishna project area *viz.,* KMP, VBP, TNP and GTP were subjected for morphological and biochemical characterization. Morphological characterization of the isolates was carried out by comparing the colony size, shape, nature of colony margin, appearance and colour of the colony and cell morphology size and shape of the cells and cell morphology with that of the reference *B. thuringiensis* subsp. *kurstaki* (HD1). The isolates were also subjected to various biochemical tests (Voges-Proskauer reaction, Citrate utilization test, Nitrate reduction, Catalase test, Arginine dihydrolysis, Starch hydrolysis, Casein hydrolysis, Esterase activity, Oxidase test, Fermentation of carbohydrates (Sucrose, glucose, trehalose hydrolysis)) as per the standard procedures in comparison with the reference strain HD1.

All 22 isolates were used for bioassays to ascertain their insecticidal activity against *P. xylostella.* The culture of *P. xylostella* was mass reared in the laboratory on mustard seedlings. The native Bt isolates was grown in the modified glucose medium (MGM) on rotary shaker at 30°C for five days until the majority of the cells had sporulated (> 95% free spores). Suspension of five day old culture was centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was re-suspended in sterile distilled water. Pellet containing crystals and spores were diluted with 20 ml sterile distilled water to conduct initial bioassay. The population count was taken by following standard serial dilution and plate count technique. Mustard leaves brought from green house, surface sterilized with 0.1 per cent sodium hypochlorite solution. Such leaves were dipped 5 minutes in cell suspension with the population of 1.2×10^6 cfu/ml, air dried aseptically and kept in separate plastic container (10 cm diameter). Ten larvae were released to each container, with three replications maintained for each isolate. The number of dead larvae was recorded at 24, 48 and 72 hr after treatment. Leaf dipped in sterile distilled water was maintained as untreated control. The data generated from the laboratory experiments were subjected to statistical analysis by Completely Randomized Design. After suitable transformation, data were subjected to analysis of variance and means were separated by Duncan's Multiple Range Test (DMRT).

Results and discussion

Eighteen soil samples were collected from eleven different cropping ecosystems. Based on the production of parasporal crystal eighteen Bt isolates obtained were after examining the 212 Bt like colonies. Out of them two from sugarcane, two from pigeonpea, one from coconut, two from mango, two from Sapota, one from fig, three from lime, two from ber, one from pomegranate, two from mulberry and none of the Bt isolates obtained from paddy ecosystem (Table 1). The present results showed low abundance of Bt in these soil samples which might be due to variation in environment, cultivated soils, dry weather and less organic matter in cropping ecosystem in contrast to undisturbed soils of forest, mountains and conifers etc. These results are well with the range of 1.60 to 14.08 per cent suggested by earlier literatures *viz.,* Thaphan *et al.* (2008), Goudar *et al.* (2012) and Jafari *et al.* (2013). The overall Bt index of all the isolates of different cropping ecosystem ranged from 0.05 to 0.30 presented in Table 1, where in Saravanan and Gujar (2005) reported an overall Bt index for Indian soil with 0.63.

All 22 isolates were characterized using morphological and biochemical tests. Eleven Bt isolates exhibited eleven circular and remaining eleven showed irregular shaped colonies. Out of twenty two Bt isolates, seventeen isolates showed white colour colonies and remaining five isolates formed creamy white colonies. All Bt isolates were found to be rough colonies with wavy margin, similar to that of the reference strain HD1 (Table 2). The diameter of the colony formed by the native Bt isolates were ranged from 3-7 mm, whereas in reference strain HD1, it was found to be 8 mm. All Bt isolates exhibited rod shaped and the average dimension was ranged from 2.5 µm x 1.0 µm to 5.0 µm x

Table 1. Details of cropping ecosystem, sampling sites, type of samples used and number of *Bacillus thuringiensis* isolates obtained from different cropping ecosystem

Cropping ecosystem	Name of the place	Type of samples	Total no of suspected colonies	No of isolates positive for Bt	Bt index of positive isolates	Isolate no.
Paddy	Gangavathi	Soil	6	0	0.00	-
	Mandya	Soil	5	0	0.00	-
	Bheemarayanagudi	Soil	13	0	0.00	-
Pigeonpea	Gulbarga	Soil	10	2	0.20	GBP-1
						GBP-2
Sugarcane	Mandya	Soil	9	2	0.22	MDS-1
						MDS-2
Coconut	Mandya	Soil	8	1	0.12	MDC
Mango	Raichur	Soil	13	0	0.00	-
	Bheemarayanagudi	Soil	18	2	0.11	BGM-1
						BGM-2
Sapota	Bheemarayanagudi	Soil	5	0	0.00	-
	Gundhalli	Soil	10	0	0.00	-
	Kavadimatti	Soil	8	2	0.25	KMS-1
						KMS-2
Fig	Gundhalli	Soil	16	0	0.00	-
	Kavadimatti	Soil	12	1	0.08	KMF
Citrus	Bheemarayanagudi	Soil	10	3	0.30	BGC-1
						BGC-2
						BGC-3
Ber	Gundhalli	Soil	18	2	0.11	GHB-1
						GHB-2
Pomegranate	Bheemarayanagudi	Soil	12	0	0.00	-
	Gundhalli	Soil	18	1	0.05	GHP
Mulberry	Raichur	Soil	13	2	0.15	RCM-1
						RCM-2
Total		18	212	18	-	18

Table 2. Colony and cell morphology of collected *Bacillus thuringiensis* isolates of different cropping ecosystem

Isolate No.	Colony morphology					Cell morphology	
	Shape	Colour	Size (diameter) (mm)	Nature	Margin	Size (ìm) (L×B)	Shape
HD-1(ref)	Circular	White	8	Rough	Wavy	5.0x1.5	Rods
MDS-1	Irregular	White	7	Rough	Wavy	4.5x1.5	Rods
MDS-2	Circular	White	3	Rough	Wavy	3.5x1.5	Rods
GBP-1	Irregular	White	4	Rough	Wavy	3.0x1.0	Rods
GBP-2	Circular	White	5	Rough	Wavy	3.0x1.5	Rods
MDC	Irregular	White	4	Rough	Wavy	3.0x1.0	Rods
BGM-1	Irregular	White	7	Rough	Wavy	4.5x1.0	Rods
BGM-2	Irregular	White	6	Rough	Wavy	4.0x1.0	Rods
KMS-1	Circular	Creamy white	3	Rough	Wavy	4.0x1.0	Rods
KMS-2	Circular	Creamy white	3	Rough	Wavy	4.5x1.5	Rods
KMF	Irregular	White	7	Rough	Wavy	2.5x1.0	Rods
BGC-1	Irregular	White	7	Rough	Wavy	5.0x1.0	Rods
BGC-2	Irregular	White	7	Rough	Wavy	4.5x1.0	Rods
BGC-3	Irregular	White	6	Rough	Wavy	4.0x1.5	Rods
GHB-1	Circular	White	4	Rough	Wavy	2.5x1.5	Rods
GHB-2	Circular	Creamy white	4	Rough	Wavy	3.5x1.5	Rods
GHP	Circular	Creamy white	4	Rough	Wavy	4.0x1.0	Rods
RCM-1	Circular	White	3	Rough	Wavy	3.5.x1.5	Rods
RCM-2	Circular	White	6	Rough	Wavy	4.5x1.5	Rods
KMP	Circular	White	4	Rough	Wavy	3.0 x1.0	Rods
VBP	Irregular	White	5	Rough	Wavy	2.5 x1.0	Rods
TNP	Circular	Creamy white	3	Rough	Wavy	3.5 x1.5	Rods
GTP	Irregular	White	6	Rough	Wavy	3.5 x1.0	Rods

1.0 µm (Table 2). Chatterjee *et al.* (2006) characterised Bt isolates collected from different regions, isolates showed varied crystal proteins and measuring more than 0.9 ìm width. Further, different colony morphology was observed among the collected isolates, indicating high variability amongst the cultures. Thus, Bt isolates obtained in this work represents, a highly diverse set of isolates even from the morphological point of view. Similar work was carried out by Monnerat *et al.* (2005), who characterized Bt strains isolated from different regions based on morphological, biochemical and molecular methods.

All 22 isolates collected in the present study from different locations were found to be Gram positive, endospore forming and parasporal crystals with a greater degree of diversity among Bt isolates distributed in soils. This has been further supported when examined for the crystal morphology under light and phase contrast microscopy. MDS-1, GBP-2, BGM-1 and BGH-2 isolates produced two types of crystals by single isolate as observed by Ozturk *et al.* (2008). Among the isolates collected from eleven different cropping ecosystem, eight isolates produced irregular shaped crystals with 36.36 per cent, followed by six isolates with spherical type (27.27%), four with both spherical and cuboidal type (18.18%), three with only cuboidal type (13.63%) and one with bipyramidal (4.5%) shaped crystals (Table 3) through some minor variations observed with the reports by Aramideh *et al.* (2010) and Thaphan *et al.* (2008) where majority of strains produced were bipyramidal crystals and ranged from 51 to 58 per cent. The great diversity observed in crystal morphology for a given isolate and between isolates could be related to the presence of novel endotoxins. These isolates may contain insecticidal proteins with specificity towards other insects groups as reported by Arrieta *et al.* (2004).

All 22 isolates were subjected to a set of biochemical test for the rapid identification of Bt isolates. Minor variation in the biochemical reaction of bacteria to biochemical test could occasionally be observed and are negligible. Totally twelve different biochemical tests were conducted to know the reaction of the Bt isolates and gave a positive reaction to V-P reaction, nitrate reduction, catalase production, casein hydrolysis, oxidase test and carbohydrate fermentation like glucose, sucrose, trehalose test. But all isolates were found to be negative for arginine hydrolys and esterase activity as shown in Table 4. Similar observations have been made by Kaur *et al.* (2006) and Goudar *et al.* (2012) who reported that the strains of Bt, besides producing parasporal crystal bodies, were positive for catalase production, oxidase activity, nitrate reduction, starch and casein hydrolysis. Totally four isolates *viz.,* MDS-2, KMS-2, KMF,

Table 3. Shape of crystals of collected *Bacillus thuringiensis* isolates

Crystal shape	Isolates	No of isolates	Per cent isolates
Bipyramidal	KMP	01	4.50
Cuboidal	BGC-2,BGC-3, GTP	03	13.63
Irregular	MDC, KMS-2, KMF, BGC-1, GHB-2, GHP-1, RCM-1, RCM-2	08	36.36
Spherical	MDS-2,GBP-1, KMS-1, GHB-1, VBP, TNP	06	27.27
Spherical and Cuboidal	MDS-1, GBP-2, BGM-1, BGM-2	04	18.18
Total		22	100

Table 4. Biochemical reactions of *Bacillus thuringiensis* isolates obtained from different cropping ecosystem

Tests	Native isolates from different cropping ecosystem																					
	HD-1	MDS-1	MDS-2	GBP-1	GBP-2	MDC	BGM-1	BGM-2	KMS-1	KMS-2	KMF	BGC-1	BGC-2	BGC-3	GHB-1	GHB-2	GHP	RCM-1	RCM-2	KMP	VBP	TNP	GTP
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+
Casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Positive reaction, - = Negative reaction

GTP, were found to be negative for starch hydrolysis. However, *B. cereus* which usually do not degrade starch are known to produce emetic toxins. Three isolates MDC, TNP and GTP contributed 13.63 per cent was found negative for citrate utilization. It could be due to absence of enzyme citrase by the organism, which is essential for utilization of carbon. In all, there was presence of variability among the isolates tested. Bioassay study was conducted with all the twenty two isolates against the third instar larvae of *P. xylostella* and the results are presented in Table 5. In general the per cent mortality increased with incubation period and maximum mortality was recorded at 72 hrs after feeding.

The mortality after 24 hr of exposure of third instar larvae of *P. xylostella* ranged from 0 to 16.67 per cent. There was no mortality observed in the untreated larvae and average mortality produced by the reference strain HD1 was 10.00 per cent. Among the native isolates, GBP-2 recorded in the highest mortality of 16.67 per cent followed by the isolates BGM-1 and KMP (13.33%) which were on par with each other. The isolates MDS-1 and RCM-2 affected 10.00 per cent mortality followed by the isolates BGM-2, KMF and RCM-1 (all with 6.67 %). The isolates MDC, BGC-3, GHB-1 and VBP were on par with each other with only 3.33 per cent mortality. No mortality was brought about by the isolates *viz,* MDS-2, GBP-1, KMS-1, KMS-2, BGC-1, BGC-2, GHB-2, GHP, TNP and GTP as well as by the control at the end of 24 hrs of feeding. In mortality was very low up to 24 hrs of feeding. This might be due to the fact that Bt being stomach poison, it has to enter in to the midgut of insect where it gets dissolved in the alkaline pH, releasing delta endotoxin, which may take more than 24 hrs time to kill the insect.

At 48 hrs after treatment cumulative mortality of *P. xylostella* ranged from 6.67 to 43.33 per cent among native isolates of Bt. While the reference strain HD1 had 100.00 per cent mortality even at 24 hrs of feeding and was significantly superior over all the isolates tested. Among the native isolates, the highest mortality (43.33%) was brought about with the isolate KMP followed by the isolates GBP-2 and BGM-2 showed 36.67 per cent mortality and both were on par with each other. The least mortality of 6.67 per cent was recorded with the isolates KMS-2, TNP and GTP. No mortality was observed in control even at the end of 48 hrs of exposure.

The mortality was ranged from 13.33 to 90.00 per cent at 72 hrs after feeding. Among the native isolates GBP-2 and BGC-1 exhibited 90.00 per cent mortality and was followed by the isolates BGC-2 (86.67%), BGM-2 (83.33%) and KMP (83.33%) which were on par with each other. Appreciable level of mortality of 73.33 per cent was recorded by the isolate BGM-1 followed by the isolates GHB-2 (63.33%), MDS-1 (53.33%), VBP (43.33%), RCM-2 (40.89%), KMF (40.00%), BGC-3 (40.00%), GHB-1 (40.00%), MDC (36.67%), RCM-1 (36.67%), MDS-2 (33.33%) and GBP-1 (30.00%). Other isolates which could affect mortality of less than 30 per cent included KMS-1 (26.67%), KMS-2 (23.33%), GHP (20.00%) and GTP (16.67%). The lowest mortality of 13.33 per cent was by the isolate TNP. Control treatment recorded zero mortality. Elevated larvicidal activity against *P. xylostella,* by the isolates may be due to the presence of multiple *cry* genes and possible synergistic activity among the *cry* proteins as reported by Xavier *et al.* (2007) and also the presence of specific binding site reported by Knowles (1994) which are to be confirmed by further investigation. The study was concluded that among different isolates tested GBP-2 and BGC-1 showed highest mortality against *P. xylostella.* Further, investigation on field efficacy should be conducted, so that they can be considered as potential candidate to control lepidopteron pests infesting agriculture crops in Northern Karnataka.

Table 5. Initial bioassay of *Bacillus thuringiensis* isolates against third instar larvae of *Plutella xylostella*

Isolates	Per cent mortality of larvae		
	24 hrs	48 hrs	72 hrs
HD-1 (ref)	10.00 (18.43)^{bc}	100.00 (90.00)^{a}	100.00 (90.00)^{a}
MDS-1	10.00 (18.43)^{bc}	26.67 (31.09)^{e}	53.33 (46.91)^{f}
MDS-2	0.00 (0.00)^{e}	13.33 (21.42)^{gh}	33.33 (35.26)^{ij}
GBP-1	0.00 (0.00)^{e}	10.00 (18.43)^{hi}	30.00 (33.21)^{jk}
GBP-2	16.67 (24.09)^{a}	36.67 (37.27)^{c}	90.00 (71.57)^{b}
MDC	3.33 (10.52)^{d}	10.00 (18.43)^{hi}	36.67 (37.27)^{hi}
BGM-1	13.33 (21.42)^{ab}	33.33 (35.26)^{c}	73.33 (58.91)^{d}
BGM-2	6.67 (14.96)^{c}	36.67 (37.27)^{hi}	83.33 (65.91)^{c}
KMS-1	0.00 (0.00)^{e}	10.00 (18.43)^{hi}	26.67 (31.09)^{kl}
KMS-2	0.00 (0.00)^{e}	6.67 (14.96)^{i}	23.33 (28.88)^{lm}
KMF	6.67 (14.96)^{c}	16.67 (24.09)^{fg}	40.00 (39.23)^{gh}
BGC-1	0.00 (0.00)^{e}	30.00 (33.21)^{de}	90.00 (71.57)^{b}
BGC-2	0.00 (0.00)^{e}	10.00 (18.43)^{hi}	86.67 (68.58)^{bc}
BGC-3	3.33 (10.52)^{d}	13.33 (21.42)^{gh}	40.00 (39.23)^{gh}
GHB-1	3.33 (10.52)^{d}	10.00 (18.43)^{hi}	40.00 (39.23)^{gh}
GHB-2	0.00 (0.00)^{e}	20.00 (26.57)^{f}	63.33 (52.73)^{e}
GHP	0.00 (0.00)^{e}	10.00 (18.43)^{hi}	20.00 (26.57)^{mn}
RCM-1	6.67 (14.96)^{c}	13.33 (21.42)^{gh}	36.67 (37.27)^{hi}
RCM-2	10.00 (18.43)^{bc}	20.00 (26.57)^{f}	41.23 (39.95)^{gh}
KMP	13.33 (21.42)^{ab}	43.33 (41.17)^{b}	83.33 (65.91)^{c}
VBP	3.33 (10.52)^{d}	20.00 (26.57)^{f}	43.33 (41.17)^{g}
TNP	0.00 (0.00)^{e}	6.67 (14.96)^{i}	13.33 (21.42)^{o}
GTP	0.00 (0.00)^{e}	6.67 (14.96)^{i}	16.67 (24.09)^{no}
Control	0.00 (0.00)^{e}	0.00 (0.00)^{j}	0.00 (0.00)^{p}
S.Em.±	1.071	0.997	0.952
C.D. at 1%	4.062	3.782	3.612

Values in the parentheses are arcsine transferred values,
The values represented by same alphabet are statistically on par with each other by DMRT mean of three replications.

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