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Bioefficacy of *Bacillus thuringiensis* against cabbage butterfly, *Pieris brassicae*

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Abstract
The present investigation was conducted to test the bioefficacy of *Bacillus thuringiensis* against cabbage butterfly, *Pieris brassicae* (L.). Thirteen *B. thuringiensis* isolates including six standard isolates (MTCC 868, MTCC 4715, 4D4, 4J3, PDBC Bt-1 and PDBC Bt-2) and seven native isolates (B1, B2, B3, B4, B5, B9 and B10) were evaluated for the morphological characteristics and further tested their efficacy against second instar larvae of *P. brassicae* (L.) in Biocontrol Laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana during the cabbage crop season in 2013-14. Different concentrations of spore crystal complex of *B. thuringiensis* isolates (250, 500 and 750µg/ml) were studied along with one commercial formulation of *B. thuringiensis* var *kurstaki* (DELFIN® WG). It was found that DELFIN®WG recorded maximum mortality of 99.98 per cent against larvae of *P. brassicae*. Among procured and native isolates, MTCC 868 and B4 recorded maximum mortality of 88.87 and 74.43 per cent, respectively. However, all treatments were significantly better than untreated control.

Keywords: *Bacillus thuringiensis*, Bioassay, Delfin, Spore Crystal, *Pieris brassicae*

1. Introduction
The cabbage butterfly, *Pieris brassicae* Linnaeus (Lepidoptera: Pieridae), is one of the important insect pests of crucifer crops including broccoli, brussel, sprouts, cabbage, cauliflower and other important crops. In India, it causes 40 per cent of the damage to cruciferous crops per year [11]. The young caterpillars feed gregariously on leaves, defoliating the plants and making insecticidal applications necessary for the cultivation of crucifers. The adverse environmental effects associated with the heavy use of chemical insecticides results in acceptance of an alternative method for insect pest control. One of the promising alternatives is the biological control which is emerging as an important component of pest management [5]. The Gram-positive bacterium *Bacillus thuringiensis* stands out representing approximately 95% of microorganisms used in biological control of agricultural pests in different cultures [16]. It synthesizes crystalline parasporal inclusions composed of insecticidal proteins, known as δ-endotoxins which when ingested by insects, gets solubilized in the midgut of insects under alkaline conditions leading to cell disruption and insect death [6, 20]. Besides the economic aspect and the safety to human health, this bacterium is the most promising for the production of biopesticides and plant resistant to insects, safe for predators and other nontarget insects [19, 22] and does not cause environmental pollution [23]. *B. thuringiensis* has been successfully used to control lepidopterous pests on a variety of crops [10], including cabbage butterfly, *Pieris brassicae* (L.) [1].
In the present study, *B. thuringiensis* isolates were characterized morphologically and also observed microscopically, further production of spore crystal and tested its toxicity against the larvae of *Pieris brassicae.*

2. Materials and Methods
The present study was conducted in Biocontrol Laboratory, Department of Entomology, Punjab Agricultural University, Ludhiana during 2013-14 in order to study the morphological characteristics of bacterial isolates and further, tested its bioefficacy against cabbage caterpillar, *Pieris brassicae.*

2.1 *B. thuringiensis* sample collection
Out 13 isolates, 6 isolates of *B. thuringiensis* procured from different sources, i.e. MTCC 868 and MTCC 4715 (Microbial Type Culture Collection and Gene Bank, IMTECH, Chandigarh.),

PDBC Bt-1 and PDBC Bt-2 (Project Director, National Bureau of Agriculturally important insects (NBAII), formerly known as Project Directorate of Biological Control, Bengluru.), Bt-4D4 and Bt-4J3 (Dr. Daniel R. Zeigler, Bacillus Genetic Stock Centre, Ohio State University, Columbus, USA) and 7 native bacterial cultures isolated from soil collected from different field crops of PAU, Ludhiana, during 2014-15 were used in the present study.

2.2 Morphological Characterization

All bacterial isolates were evaluated for the morphological characteristics like colony morphology, gram reaction, motility test, spore staining and observed under microscope. Spore staining was done as per the method of Jisha and Benjamin [12]. Bacterial smear was prepared on a clean glass slide, air dried and heat fixed. The smear was with malachite green and placed on a boiling water bath for 10 min. The slide was replaced, cooled and washed under running tap water. The smear was counter stained with safranin for 1 min. The slide was washed with tap water, blot dried and examined under the microscope. Further, motility of bacterial isolates was also tested using method described by Aygan and Arikan [4]. The motility media was prepared using Beef Extract (10g), Peptone (10g), Sodium chloride (5g) and Agar (4g) per litre distilled water. The motility media tubes were stab-inoculated with a straight wire to the depth of about 5mm and incubated at 30 °C for 24-48 hrs and observed for cloudiness in the medium. The motile bacteria moved away from the line of inoculation and showed cloudiness in the medium.

2.3 Insect Rearing

Pieris brassicae larvae and eggs were obtained from cabbage/brassica field of the Entomological Research Farm, Punjab Agricultural University, Ludhiana and were reared on natural diet in the Biocontrol Laboratory, Department of Entomology, PAU, Ludhiana. The second instar larvae of *P. brassicae* were used in the present studies.

2.4 Bioassay Study

Spore crystal toxin complex (SCT) of all the isolates was prepared by following the method of Ammouneh, *et al.* [2] with some modifications. In 500ml flasks, 100ml of T3 medium was inoculated with 1 loop of bacteria and shaken at 200 rpm for 5 days at 30°C. At the end of this incubation, the majority of the population was in the form of spore crystals as monitored by light microscope. The bacterial spore crystal mixture was harvested in the form of pellet by centrifugation at 10,000 rpm for 15 minutes. The pellet was washed twice with sterile distilled water and centrifuged at 10000 rpm for 10 min. Pellets were then processed through a freeze drier and resulting dry powders were estimated for protein content by Lowry, *et al.* [18]. Different concentrations of spore crystal complex of *B. thuringiensis* (250, 500 and 750 µg/ml) were prepared by dissolving required amount of spore crystal dried powder in sterilized distilled water and were assayed for biological activity.

Leaf dip bioassay method was used to study the insecticidal activity of *B. thuringiensis* isolates [13]. The washed leaves were dipped for 5-10 seconds in solution of different concentrations of spore crystal complex and allowed to dry and then placed individually in plastic vials. Ten larvae were introduced in each vial and there were three replications per treatment. The larvae were allowed to feed on treated leaves. The mortality was recorded after fifth, seventh and tenth days of treatment.

2.5 Statistical analysis

The statistical analysis was done as per Complete Random Design (CRD) by using one way analysis of variance (ANOVA).

3. Results

3.1 Morphological Characterization

In present study, all the 13 isolates were morphologically characterized based on two different selective media i.e. Luria agar and T3 media. On Luria agar, all the isolates formed circular, entire, flat colonies of creamish to off white colour after 48 h of incubation at 30°C. Bacterial isolates showed circular creamish to off white coloured with smooth, mucoid and fried egg like appearance on T3 media. These cultures were further identified based on Gram staining and it was observed that all the tested isolates were gram positive with rod shaped in short to long chains. The isolates were also tested for their motility by stab inoculation method and found that all the isolates were motile except native isolate B5. It was also observed that all isolates produced spore except 5 native isolate namely B1, B2, B3, B5 and B9.

3.2 Bioassay studies

Bio-efficacy of tested isolates and commercial formation Delfin was evaluated against second instar larvae of *P. brassicae*. The maximum mean cumulative per cent mortality at lower concentration (250 µg/ml) after 5th day of treatment was recorded 43.33 per cent in MTCC 868 against second instar larvae of *P. brassicae* at lower concentration (250 µg/ml) of spore crystal complex which was at par with all the other procured isolates except PDBC Bt-1 (30.00%) and 4J3 (16.66%) and was also at par with local isolate B4 with 33.33 per cent mortality (Table 1). However, commercially available *B. thuringiensis* DELFIN® WG recorded mean cumulative per cent mortality of 99.98 per cent which was significantly better than all other treatments at all the concentrations.

Similarly, at middle concentration (500 µg/ml), isolate MTCC 868 recorded maximum mortality of 56.66 per cent and was at par with MTCC 4715 (53.33%) and local isolate B4 (43.33%). At higher concentration (750 µg/ml), 70.00 per cent mortality was recorded in case of local isolate B4 and was at par with MTCC 868 (66.66%), MTCC 4715 (63.33%), 4D4 (63.33%) and local isolate B10 (60.00%).

The commercial formulation Delfin was significantly better than all the treatments at all the concentrations after seven days of treatments with 99.98 per cent mortality and was at par with isolate MTCC 868 which recorded 96.65 per cent mortality at higher concentration (Table 2). The isolate MTCC 868 recorded maximum mortality at all the concentrations and was at par with local isolate B4 and all the procured isolates except 4J3 at both lower and middle concentration and with isolate MTCC 4715 at higher concentration.

After ten days of treatment, all the procured isolates except 4J3, and local isolates B4 and B10 were recorded similar insecticidal activity and were at par with each other and with commercial formulation Delfin at higher concentration (Table 3). All the treatments were significantly better than untreated control.

4. Discussion

The results of present study were in corroboration with the findings of Lenina, *et al.* [17] who observed that the seven isolates of *B. thuringiensis* produced creamy white colonies

after 24h of inoculation on T3 agar plates and formed flat or raised colonies with entire or undulated margin. Other studies also showed white, round, flat colonies of *B. thuringiensis* isolates with irregular but entire margin on nutrient agar medium and some isolates also showed fried egg appearance [9]. Motility of *B. thuringiensis* isolates were also tested on nutrient agar plates and were reported as motile [5]. Non-motile *B. thuringiensis* had also been reported [8]. Shishir, *et al.* [21] identified fifty seven isolates as *B. thuringiensis*, out of which fifteen isolates were found to be sporulating when observed under Phase contrast microscope and parasporal crystal proteins were also observed. All the bacteria were rod shaped and were positive for gram stain, spore and crystal staining [7].

In the present studies, native isolate B4 showed 99.98% mortality at higher concentration (750µg/ml) after 10 days of treatment as reported in studies of Anandhi, *et al.* [3] where five native strains, NSC-1, NSC-3, NSC-9, COR-4 and GUR-5 emerged as novel isolates for controlling important lepidopteran pests of cabbage. These five isolates showed high toxicity against pests with 100% mortality. Further, some of the native isolates used in present studies recorded more than 50% mortality after 10 days. It was in accordance with the studies of Khodabandeh, *et al.* [15] who conducted bioassay test on *P. brassicae* and *Ephestia kuehniella* (Zeller) with native isolates and recorded that 28.57 and 14.28 per cent of the isolates were toxic to the larvae of *P. brassicae* and *E. kuehniella*, respectively, causing more than 50% mortality.

Table 1: Efficacy of *B. thuringiensis* isolates for the control of second instar larvae of *P. brassicae* five days after treatment

Treatments	Cumulative per cent mortality (Different concentrations of spore crystal complex)		
	250µg/ml	500µg/ml	750µg/ml
MTCC 868	43.33 (41.05)	56.66 (48.82)	66.66 (54.96)
MTCC 4715	40.00 (39.21)	53.33 (46.90)	63.33 (52.75)
4D4	36.66 (37.20)	40.00 (39.13)	63.33 (52.75)
4J3	16.66 (23.84)	23.33 (28.76)	43.33 (41.05)
PDBC Bt-2	40.00 (39.21)	40.00 (39.13)	56.66 (48.82)
PDBC Bt-1	30.00 (32.98)	36.66 (37.20)	50.00 (44.98)
B1	13.33 (21.13)	26.66 (30.98)	43.33 (41.13)
B2	16.66 (23.84)	23.33 (28.76)	23.33 (28.76)
B3	13.33 (21.13)	16.66 (23.84)	23.33 (28.76)
B4	33.33 (35.20)	43.33 (41.13)	70.00 (56.97)
B5	13.33 (21.13)	26.66 (30.98)	33.33 (35.20)
B9	13.33 (21.13)	13.33 (21.13)	16.66 (23.84)
B10	16.66 (23.84)	33.33 (35.20)	60.00 (50.83)
Delfin WG	99.98 (89.15)	99.98 (89.15)	99.98 (89.15)
Control	0.00 (0.80)	0.00 (0.80)	0.00 (0.80)
LSD (p=0.05)	(7.00)	(8.24)	(7.67)

Figures in parentheses are the mean of arc sine √percentage transformations

Table 2: Efficacy of *B. thuringiensis* isolates for the control of second instar larvae of *P. brassicae* seven days after treatment

Treatments	Cumulative per cent mortality (Different concentrations of spore crystal complex)		
	250µg/ml	500µg/ml	750µg/ml
MTCC 868	56.66 (48.91)	70.00 (56.97)	96.65 (83.28)
MTCC 4715	50.00 (44.98)	66.66 (54.76)	80.00 (63.90)
4D4	43.33 (41.05)	56.66 (48.82)	76.66 (61.19)
4J3	30.00 (32.98)	46.66 (43.05)	63.33 (52.75)
PDBC Bt-2	50.00 (44.98)	60.00 (50.83)	76.66 (61.19)
PDBC Bt-1	43.33 (41.13)	60.00 (50.83)	63.33 (52.75)
B1	23.33 (28.76)	40.00 (39.13)	56.66 (48.82)
B2	26.66	33.33	40.00 (39.13)
B3	(30.77)	(34.99)	30.00 (32.98)
B4	16.66 (23.84)	26.66 (30.98)	69.96 (55.57)
B5	53.33 (46.90)	60.00 (50.83)	40.00 (39.13)
B9	13.33 (21.13)	33.33 (35.20)	36.66 (37.20)
B10	16.66 (23.84)	26.66 (30.98)	76.66 (61.19)
Delfin WG	26.66 (30.77)	46.66 (43.05)	99.98 (89.15)
Control	99.98 (89.15)	99.98 (89.15)	3.34 (6.98)
	0.00 (0.80)	0.00 (0.80)	
LSD (p=0.05)	(9.38)	(8.85)	(19.65)

Figures in parentheses are the mean of arc sine √percentage transformations

Table 3: Efficacy of *B. thuringiensis* isolates for the control of second instar larvae of *P. brassicae* ten days after treatment

Treatments	Cumulative per cent mortality (Different concentrations of spore crystal complex)		
	250µg/ml	500µg/ml	750µg/ml
MTCC 868	70.00 (56.97)	96.65 (83.28)	99.98 (89.15)
MTCC 4715	66.66 (54.76)	93.32 (77.40)	99.98 (89.15)
4D4	50.00 (44.98)	63.33 (52.75)	99.98 (89.15)

4J3	43.33 (41.13)	56.66 (48.82)	86.66 (72.48)
PDBC Bt-2	60.00 (50.83)	80.00 (63.90)	99.98 (89.15)
PDBC Bt-1	46.66 (43.05)	86.66 (72.48)	96.65 (83.28)
B1	53.33 (46.90)	63.33 (52.75)	76.66 (61.19)
B2	30.00 (32.98)	46.66 (43.05)	56.66 (48.91)
B3	30.00 (32.98)	43.33 (41.13)	43.33 (41.13)
B4	56.66 (48.82)	66.66 (54.76)	99.98 (89.15)
B5	16.66 (23.84)	36.66 (37.20)	43.33 (41.13)
B9	33.33 (35.20)	36.66 (37.20)	46.66 (43.05)
B10	33.33 (35.20)	60.00 (50.83)	86.66 (72.48)
Delfin WG	99.98 (89.15)	99.98 (89.15)	99.98 (89.15)
Control	0.00 (0.80)	0.00 (0.80)	0.00 (0.80)
LSD (p=0.05)	(7.34)	(11.00)	(10.71)

Figures in parentheses are the mean of arc sine √percentage transformations

5. Conclusion

In this study, 13 isolates of *B. thuringiensis* characterized based on their morphology and motility and further evaluated for their insecticidal activity on second instar larvae. The insecticidal activity of local isolate B4 was impressive as it was very close to that of standard isolate MTCC 868. However, efficacy of all the isolates was less as compared to the commercial formulation Delfin.

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