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Morphological and biochemical characterization of *Bacillus thuringiensis* Berliner isolates and their evaluation against *Plutella xylostella* Linnaeus

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ABSTRACT: **Eleven isolates of *Bacillus thuringiensis* were morphologically and biochemically characterized and evaluated for insecticidal activity against *Plutella xylostella* Linnaeus under field-cum-laboratory conditions. All the isolates were gram positive, rod shaped, spore forming and showed typical colony morphology with mucoid or glistening surfaces having entire edges and density ranging between translucent to opaque. Glucose was fermented by all the cultures only with acid production. Xylose and Mannitol were fermented by *Bt* d and *Bt* VK isolates, respectively. All cultures were catalase positive, oxidase positive, nitrate reducing (except *Bt* d), degraded tyrosine, hydrolysed casein and starch and utilized citrate as a carbon source. Results of bioassay studies revealed that four isolates, namely, *Bt* 5, 4D4, *Bt* 9 and MTCC 868 caused complete mortality of the second instar larvae of *Plutella xylostella* within 96 hours at uniform concentration of 10^9 spores/ml.**

KEY WORDS: *Bacillus thuringiensis*, bioassay, insecticidal activity, *Plutella xylostella*

INTRODUCTION

The control of insect population by entomopathogenic microorganisms today is an important alternative to chemical insecticides. *Bacillus thuringiensis* (*Bt*), a soil bacterium is being widely used as biopesticidal formulation to control pest population among Lepidoptera, Diptera and Coleoptera (Schnepf *et al.*, 1998). There are more recent reports of *B. thuringiensis* isolates active against other insect orders such as Hymenoptera, Homoptera and Orthoptera (Garcia-Robeles *et al.*, 2001). Diamondback moth, *Plutella xylostella* Linnaeus is most serious pest of cabbage and cauliflower and is responsible for low productivity of these crops in India (Verma and Sandhu, 1968), and *Bt* isolates have been found to be quite effective against this pest. The management of this pest has become difficult, as it has developed resistance to almost all the available insecticides (Mehrotra, 1991). In the present investigation, different *Bacillus thuringiensis* Berliner isolates were characterized for their morphological and biochemical properties and were evaluated for their insecticidal activity

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against *P. xylostella* under field-cum-laboratory conditions.

MATERIALS AND METHODS

Eleven cultures of *B. thuringiensis* isolates *i.e.* standard as well as local isolates were used in the present study.

Characterization of *Bacillus thuringiensis* isolates

All the Bt isolates were characterized on the basis of morphological and biochemical tests. They were observed on the basis of morphology, colony characteristics, gram's reaction and cell size. The various biochemical tests, namely, carbohydrate fermentation test, catalase test, oxidase test, citrate utilization test, nitrate reduction test, casein and starch hydrolysis, decomposition of tyrosine were performed as per their standard methods.

Bioassay studies

The bioassay studies were conducted at Entomology Farm and Microbiological Laboratory of Biocontrol Unit of Department of Entomology, Punjab Agricultural University, Ludhiana. The recommended variety of cabbage 'Pride of India' was sown in September, 2003 at Entomological Research Farm. The larvae of *P. xylostella* were collected from the cabbage crop and were brought to the biocontrol laboratory for multiplication. The culture of *P. xylostella* was reared in the laboratory and was maintained on cabbage leaves. Second instar larvae were used for bioassay.

Initially, all the eleven *B. thuringiensis* isolates were inoculated on Luria agar and incubated at 30±2°C for 48 hours. The growth was harvested in 10 ml aliquots of sterile distilled water aseptically. These suspensions were then mixed well and 10 ml of each inoculum was added to 250 ml capacity flasks and were incubated undisturbed as static cultures at room temperature. Complete sporulation was obtained on the fifth day. The spore-crystal mixtures thus obtained were standardized based on haemocytometer count of spores and all the cultures were adjusted to carry 10^9 spores/ ml. Before using these suspensions in the bioassays, CMC-Triton X-100 was added to provide uniform distribution of spores in the suspensions.

Bt suspensions along with commercially available biopesticides *i.e.* Dipel 8L and chemical insecticide (Endosulfan 35EC) were sprayed on the cabbage crop sown at the farm. Then after 24 hours, sprayed leaves were brought to the laboratory and were fed to second instar larvae of *P. xylostella* kept in Petri-dish. The larvae were starved for 6 hours were released on treated leaves @ 10/ leaf disc. The treatments were replicated three times. Suitable control was also maintained. The larvae were screened daily for their mortality. The cumulative mortality was worked out and data were analyzed as per Completely Randomized Design (CRD).

RESULTS AND DISCUSSION

All strains were of typical colony morphology, which was predominantly of circular creamish to off white in colour with mucoid or glistening surfaces having entire edges and density ranging between translucent to opaque.

All cultures were found to be gram positive, sporulating, crystal producing and were rod shaped occurring in short to long chains. Cell size varied between 1.0 x 3.0 to 1.2 x 4.6 μm. The spore position was either central or terminal.

It was also found that all the strains fermented glucose only with acid but no gas was produced (Table 1). Other carbohydrates *i.e.* Sucrose, Maltose, Lactose, Rhamnose, Galactose were not fermented by any strain. Xylose and Mannitol was fermented by *Bt* d and *Bt* VK, respectively. All the remaining isolates did not utilize Xylose and Mannitol.

Table 1. Carbohydrate fermentation reactions of *Bacillus thuringiensis* isolates

Isolates	Carbohydrates							
	Glucose	Sucrose	Maltose	Xylose	Mannitol	Lactose	Rhamnose	Galactose
4D1	+	-	-	-	-	-	-	-
4D4	+	-	-	-	-	-	-	-
4A3	+	-	-	-	-	-	-	-
4J3	+	-	-	-	-	-	-	-
MTCC 868	+	-	-	-	-	-	-	-
MTCC 869	+	-	-	-	-	-	-	-
Bt 1	+	-	-	-	-	-	-	-
Bt 5	+	-	-	-	-	-	-	-
Bt d	+	-	-	+	-	-	-	-
Bt 9	+	-	-	-	-	-	-	-
BtVK	+	-	-	-	d	-	-	-

+ = Positive for acid production

- = Negative for acid production

d = Delayed positive reaction

All the eleven cultures were characterized on the basis of biochemical tests, results of which are presented in Table 2. All the eleven strains were found to be catalase positive, as shown by oxygen evolution on reaction with H_2O_2. Tyrosine was typically degraded by all the strains. Oxidase test was positive for all strains except MTCC 869 and Bt d, which gave delayed reactions. All strains except Bt d reduced nitrate to nitrite, which showed that these strains had ability to utilize nitrate as terminal electron acceptor in place of oxygen. As regards utilization of citrate as carbon source, they utilized citrate and showed good growth. All cultures hydrolyzed casein and starch and showed growth.

Sneath (1986) suggested that the Bt colonies show more or less pronounced differences in colour and translucence and opaqueness because of different degrees of sporulation within colonies. Smith *et al.* (1946) did not give much weightage on identification of *Bacillus* species on the basis of fermentation test. They emphasized that the *Bacillus thuringiensis* strains because of their strong proteolytic nature, may not form enough acid to overcome alkalinity produced from organic nitrogen, thus do not produce observable change in an indicator. The ability to utilize nitrate as terminal electron acceptor has been reported in number of other organisms such as *Pseudomonas* (Palleroni *et al.*, 1970).

Results of bioassay of Bt strains against second instar larvae of *P. xylostella* showed variation in response to various strains of *Bt* when they were exposed to them. Cumulative mean per cent mortality of second instar larvae ranged from 0.00 to 23.33 after 24 hours of treatment (Table 3). The mortality in Endosulfan (23.33%) was significantly higher than all other strains except MTCC 868, Bt 5, 4 D1, 4 D4 and Dipel 8L. The mortality after 48 hours of exposure ranged from 0.00 per cent in control to highest 60.00 per cent in Bt 5 strain, which was on par with 4 D4 (56.67%). The mortality after 72 hours in control was 3.33 per cent and all treatments gave significantly higher mortality than control. Complete mortality was given by Bt 5 and 4 D4, which were significantly better than chemical insecticide *i.e.* Endosufan and Biopesticide (Dipel 8L) and all other strains except

Table 2. Biochemical characteristics of *B. thuringiensis* isolates

Isolate	Biochemical characteristics						
	Catalase	Oxidase	Nitrate reduction	Tyrosine degradation	Citrate utilization	Starch hydrolysis	Casein hydrolysis
4D1	++	+	+	+	+	++	+
4D4	++	+	+	+	+	+	+
4A3	++	+	+	++	+	++	+
4I3	+	+	+	+	d	++	d
MTCC 868	++	+	+	++	+	+	+
MTCC 869	+	d	+	+	d	+	+
Bt 1	+	+	+	+	+	++	d
Bt 5	++	+	+	++	+	++	+
Bt d	+	d	-	+	+	+	+
Bt 9	+	+	+	++	+	+	+
BtVK	++	+	+	+	+	+	+

\+ Positive for acid production
Negative for acid production
d Delayed positive reaction

Table 3. Efficacy of different isolates of *B. thuringiensis* against *P. xylostella*

Isolate	Cumulative per cent mortality after			
	24h	48h	72h	96h
MTCC 868	10.00 (18.90)	50.00 (45.26)	96.33 (83.98)	100.00 (90.00)
4I3	3.33 (9.00)	43.33 (41.43)	73.33 (59.31)	76.67 (61.54)
Bt5	13.33 (21.57)	60.00 (51.04)	100.00 (90.00)	100.00 (90.00)
4A3	6.67 (13.95)	36.67 (37.51)	70.00 (57.08)	80.00 (63.77)
Btd	3.33 (9.00)	33.33 (35.51)	53.33 (47.19)	70.00 (57.08)
4D4	13.33 (21.57	56.67 (49.12)	100.00 (90.00)	100.00 (90.00)
*Bt*VK	6.67 (13.95)	43.33 (41.43)	66.67 (55.07)	76.67 (61.54)
4D1	10.00 (18.90)	43.33 (41.43)	83.33 (66.q52)	86.67 (69.27)
MTCC 869	3.33 (9.00)	30.00 (33.51)	46.67 (43.35)	60.00 (51.04)
*Bt*1	6.67 (13.95)	40.00 (39.51)	60.00 (51.04)	76.67 (61.54)
*Bt*9	6.67 (13.95)	46.67 (43.35)	90.00 (72.02)	100.00 (90.00)
Dipel 8L	10.00 (18.90)	40.00 (39.51)	80.00 (63.77)	83.33 (66.52)
Endosulfan	23.33 (29.11)	43.33 (41.43)	60.00 (51.04)	70.00 (57.08)
Control	0.00 (4.05)	0.00 (4.05)	3.33 (9.00)	3.33 (9.00)
CD (P=0.05))	(10.69)	(4.25)	(7.11)	(5.72)

Conc. = 10^9 spores/ml

Values in parentheses are arcsine transformations.

MTCC 868 (96.33%). Lowest mortality was recorded in MTCC 869 (46.67%) and it was significantly lower than all other treatments except Bt d (53.33%).

Ninety-six hours after exposure all the treatments gave significantly higher mortality than control (3.33%). Four *Bt* strains namely MTCC 868, *Bt* 5, *Bt* 9 and 4 D4 caused 100.00 per cent mortality and they were significantly better than commercially available biopesticide *i.e.* Dipel 8L and chemical insecticide *i.e.* Endosulfan 35EC and the remaining *Bt* strains.

It can be concluded that four *Bt* strains, namely, MTCC 868, *Bt* 5, *Bt* 9 and 4D4 proved very effective against second instar larvae of *P. xylostella*. Thus, the results of these pathogenicity studies of *Bt* on *P. xylostella* clearly indicated the difference in insecticidal activity among them. The results are in close agreement with that of Justin *et al.* (1989) reported the variation in insecticidal activity among the various *Bacillus thuringiensis* sub-species. The toxicity of *Bt* mainly depends upon the delta endotoxin. The delta endotoxins produced by different strains have different spectra of insecticidal activity. The present findings agree with the result obtained by Adams *et al.* (1996) who reported that the different strains are known to vary in their toxicity and different isolates of the same strain may show variable levels of toxicity against same species of insect pest. Pokharkar *et al.* (2002) reported that toxicity of *Bt* persisted at least for 120 hours on cabbage leaves. Malathi *et al.* (1999) reported that the *Bt* formulations *viz.* Dipel 8L and Delfin were very effective in reducing the population of *P. xylostella* as compared to endosulfan and other botanical insecticides. Hence, *Bt* can be incorporated as one of the tools for the management of *P. xylostella* on cabbage.

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