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Effectiveness of beta-exotoxin of *Bacillus thuringiensis* var. *thuringiensis* on the ability of *Meloidogyne* sp. from brinjal (*Solanum melongena* L.) to survive

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Abstract. Beta-exotoxin produced by *Bacillus thuringiensis* var. *thuringiensis* grown in the acid hydrolysates of wheat and rice brans caused 95% and 85% mortality respectively of *Meloidogyne* sp. as against 72% of *ß*-exotoxin produced on farm yard manure within 7 days. Acid hydrolysate of wheat or rice bran and solid farm yard manure proved to be the best media for growth of *B. thuringiensis* var. *thuringiensis.*

Keywords. *Bacillus thuringiensis* var. *thuringiensis*; *Meloidogyne* sp.; beta-exotoxin. Wheat bran; rice bran; farm yard manure.

Introduction

Plant parasitic nematodes, *Meloidogyne* sp. have been known for a long time to cause root knot diseases in various vegetable crops. These can be controlled effectively by using certain nematicides (Mankau 1961, 1962; Singh and Sitaramaiah, 1966). Although there are several reports on the use of nematophagous fungi and other natural enemies for the control of nematodes, there is a paucity of literature on soil bacterial forms parasitising on phytopathogenic nematodes. Prasad and Tilak (1972), Prasad *et al.* (1972) and Srivastava (1973) have shown that *Meloidogyne* sp. in tomato and okra can be controlled through *Bacillus thuringiensis* var. *thuringiensis.* The present investigation was undertaken to study the effect of *ß*-exotoxin of *Bacillus thuringiensis* var. *thuringiensis* on *Meloidogyne* sp. from brinjal (*Solanum melongena* L.) var. pusa purple long, and to use certain natural substrates for the growth of this bacterial culture.

Materials and methods

Organism used

The test organism *B. thuringiensis* var. *thuringiensis* No. 504–6–H was supplied by the International Minerals and Chemicals, USA. This was maintained on nutrient agar slants.

Preparation of water extracts of farm yard manure, wheat and rice brans

The farm yard manure, wheat and rice brans (test carriers) were obtained from the Experiment Station of G. B. Pant University of Agriculture and Technology, Pantnagar. Powdered test carrier (100 g) was mixed separately with 150 ml of distilled water in 500 ml Ehrlenmeyer flask, thoroughly mixed and extracted overnight at room temperature. The autoclaved filtrates were used in the experiments.

Preparation of acid hydrolysates of wheat and rice brans

Wheat and rice brans (100 g) were separately hydrolysed with 150 ml of 6 N H_2SO_4, heated for 6 h at 100° C. After cooling, the acid was neutralised by the addition of $CaCO_3$. The filtrate was used in the experiments after autoclaving.

Growth of organism on the test carriers

Farm yard manure (9 g) was taken in each of the three 100 ml Erlenmeyer flasks and were autoclaved for 2 h at 15 lb pressure. This was inoculated with 1 ml heat-shocked (80° C for 30 min) spore suspension of *B. thuringiensis* var. *thuringiensis* (1×10^4 spore| ml). The inoculated farm yard manure was thoroughly mixed by means of sterile spatula and incubated at 30 ± 1° C for 72 h and aerated for 24 h. The flask was tightly plugged with cotton and polythene. After incubation, the flasks were stored at 0–4° C until further use.

Wheat bran and rice bran sterilised as described above were used alone or in different combinations with farm yard manure, for the growth of bacterial culture. Moisture content was maintained by the addition of 3 ml of water. The following combination of carriers were evaluated for their ability to support the growth of bacterial culture: wheat bran; rice bran; farm yard manure (all 9 g); wheat bran (4·5 g) + farm yard manure (4·5 g); rice bran (4·5 g) + farm yard manure (4·5 g); and wheat bran (3 g) + rice bran (3 g) + farm yard manure (3 g). The total viable counts and heat stable counts were observed at 0 h, 72 h and 7th day in each treatment. The sterility checks were also performed.

Growth of the organism on different water extracts of test carriers and on acid hydrolysate of brans

Water extracts of wheat bran, rice bran and farm yard manure (9 ml) and acid hydrolysate of wheat bran, rice bran and combinations of these in 1 :1 or 1:1:1 ratio were inoculated with heat-shocked (80° C for 30 min) spore suspension of *B. thuringiensis* var. *thuringiensis* (1×10^4 spores/ml). The cultures were incubated on a gyratary shaker (160 rpm) for 7 days at 30 ± 1°C and were stored at 0–4° C before extraction of the crude exotoxin.

The total viable counts and heat stable counts were observed at 0, 72 h and on the 7th day in each treatment. The sterility checks were also performed. The total sugars, reducing sugars and total nitrogen contents were estimated by the Anthrone, Nelson-Somogyis' and Macrokjeldhal methods respectively (Chaykin, 1966; Hawk *et al*., 1954).

Extraction of crude β-exotoxin from B. thuringiensis var. thuringiensis grown on different test carriers

Crude *β*-exotoxin, which was produced by *B. thuringiensis* var. *thuringiensis* on different test carriers, was extracted according to the method of Prasad and Tilak (1972) and Prasad *et al.* (1972). For control preparations, the same procedure was followed except that test carriers were not inoculated with spore suspension.

Collection and preparation of sterile second stage larvae of Meloidogyne sp.

The second stage larvae of *Meloidogyne* sp. were extracted and sterilised by the method described by Srivastava (1973). Heavily infested roots (5 g) of brinjal (pusa purple long) were chopped and suspended in 50 ml of distilled water. This mixture was churned for 1 min in a Waring blendor. The suspension of macerated roots and tissue was then passed successively through 60 and 200 mesh sieves. The root tissue, which did not pass through 60 mesh sieve, was discarded and that retained on 200 mesh sieve was thoroughly washed by fine jet of water to remove plant sap and other residues. The macerated suspension was suspended in water, centrifugated at 450 g for 5 min. The supernatant was discarded and the root debris contained the free eggs was then suspended in 0·3 M NaCl solution and stored at room temperature.

For obtaining sterile second stage larvae, a plastic ring, 7 cm in diameter and 5 cm deep, was fitted with two folds of tissue paper on the inner side and covered by a piece of bandage cloth on the outside and tightly held by a rubber band. The ring was placed in a round bottom dish and the suspension of root debris and eggs in 0·3 M NaCl solution was placed on to the tissue paper. Sufficient water was poured so that the base of the ring just dips in water. This assembly was kept at 30 ± 1° C. After 4 to 5 days the eggs hatch and the hatched larvae wriggled down through the tissue paper and collected at the bottom. These evenly hatched larvae were sterilised by transferring them into a broth containing penicillin (5000 units/ml) and streptomycin (5000 units/ml) by means of a capillary tube. After 24 h, these larvae were washed with sterile distilled water and used in this study.

Laboratory trials of test suspension on the survivability of second stage larvae of Meloidogyne sp. from brinjal roots

Test suspensions (2 ml) were placed in a surface sterilised cavity glass blocks (2.54 × 2.54 cm) into which 50 sterile second stage larvae were introduced by means of a glass capillary tube. The glass blocks were covered with glass plates, and were incubated at 30 ± 1° C. In each case, three replications were used. The per cent mortality in all the treatments was observed after 1, 2 and 7 days of treatment. In the control experiment, sterile larvae were introduced into a glass block containing 2 ml of sterile distilled water.

Methods for distinguishing between live and dead nematodes

The larvae which were immobilised, after appropriate period of incubation in each test medium, were picked up under stereoscopic microscope using a glass capillary tube and transferred to another block containing 2 ml of distilled water. After 24 h the larvae which revived, were counted as living.

Results

Estimation of total carbohydrate, reducing sugars and nitrogen content in test carriers

The results presented in table 1 show that the water extracts of wheat and rice brans contained a greater amount of (27 and 24-fold) carbohydrates than reducing sugars, whereas in farm yard manure the total content of carbohydrate and reducing sugars were approximately the same. The total carbohydrate and reducing sugars were more in the acid hydrolysate of wheat bran than the acid hydrolysate of rice bran. The total nitrogen content was highest in the water extract of farm yard manure which was followed by the water extracts of wheat and rice brans. On the other hand, the total nitrogen content was more in the acid hydrolysate of wheat bran than that of rice bran acid hydrolysate.

Table 1. Estimation of total carbohydrate, reducing sugars and nitrogen content of different test carriers.

Substrate	Total carbo-hydrate content (mg/ml)	Total reducing sugars content (mg/ml)	Total nitrogen content (mg/ml)
Wheat bran[a]	37·00	1·35	0·105
Rice bran[a]	29·00	1·15	0·070
Farm yard manure[a]	50·00	47·50	0·525
Wheat bran[b]	100·00	84·00	1·575
Rice bran[b]	70·00	61·00	1·190

[a] Water extract
[b] Acid hydrolysate

Growth of B. thuringiensis var. thuringiensis on different test carriers

The wheat and rice brans separately or both together in combination with farm yard manure in a ratio of 1 :1 or 1 :1 :1 supported growth and sporulation of *B. thuringiensis* var. *thuringiensis* which was 10 times lesser than the growth and sporulation of this organism in farm yard manure. Similar results were obtained with the use of water extracts of the test carriers and their combinations (table 2). Acid hydrolysates of wheat and rice brans separately or in combination with water extract of farm yard manure resulted in better growth and sporulation of the organism which was larger than the growth and sporulation in water extract of farm yard manure (table 3).

Table 2. Effect of test carriers and their combinations on the growth of *B. thuringiensis* var.

Treatment	Solid form Counts*				Water	
	72 h		7th day		72 h	
	TVC[a]/g	HSC/g	TVC/g	HSC/g	TVC/ml	HSC/ml
Wheat bran	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$
Rice bran	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$	$< 10^4$
Wheat bran + farm yard manure	$1{\cdot}40 \times 10^8$	$1{\cdot}35 \times 10^8$	$1{\cdot}75 \times 10^8$	$1{\cdot}64 \times 10^8$	$1{\cdot}93 \times 10^7$	$1{\cdot}83 \times 10^7$
Rice bran + farm yard manure	$1{\cdot}39 \times 10^8$	$1{\cdot}28 \times 10^8$	$1{\cdot}69 \times 10^8$	$1{\cdot}55 \times 10^8$	$1{\cdot}89 \times 10^7$	$1{\cdot}81 \times 10^7$
Wheat bran + rice bran + farm yard manure	$1{\cdot}54 \times 10^8$	$1{\cdot}42 \times 10^8$	$1{\cdot}83 \times 10^8$	$1{\cdot}74 \times 10^8$	$1{\cdot}95 \times 10^7$	$1{\cdot}84 \times 10^7$
Farm yard manure	$1{\cdot}78 \times 10^9$	$1{\cdot}60 \times 10^9$	$2{\cdot}11 \times 10^9$	$1{\cdot}91 \times 10^9$	$1{\cdot}65 \times 10^8$	$1{\cdot}58 \times 10^8$

* Counts at 0 h varies between $0{\cdot}86 \times 10^4$ to $0{\cdot}91 \times 10^4$ heat stable counts/g or ml
[a] TVC Total viable counts
[b] HSC Heat stable counts

Table 3. Effect of test carriers and their combinations on the growth of *B. thuringiensis* var. *thuringiensis*.

Treatments	Counts at 72 h		Counts at 7th day	
	TVC/ml	HSC/ml	TVC/ml	HSC/ml
Wheat bran[a]	$1{\cdot}88 \times 10^8$	$1{\cdot}65 \times 10^8$	$2{\cdot}22 \times 10^8$	$2{\cdot}09 \times 10^8$
Rice bran[a]	$1{\cdot}87 \times 10^8$	$1{\cdot}62 \times 10^8$	$2{\cdot}17 \times 10^8$	$2{\cdot}02 \times 10^8$
Wheat bran[a] + farm yard manure[b]	$2{\cdot}03 \times 10^8$	$1{\cdot}94 \times 10^8$	$2{\cdot}28 \times 10^8$	$2{\cdot}19 \times 10^8$
Rice bran[a] + farm yard manure[b]	$2{\cdot}01 \times 10^8$	$1{\cdot}90 \times 10^8$	$2{\cdot}23 \times 10^8$	$2{\cdot}19 \times 10^8$
Wheat bran[a] + rice bran[a] + farm yard manure[b]	$2{\cdot}16 \times 10^8$	$2{\cdot}02 \times 10^8$	$2{\cdot}37 \times 10^8$	$2{\cdot}20 \times 10^8$
Farm yard manure[b]	$1{\cdot}65 \times 10^8$	$1{\cdot}58 \times 10^8$	$1{\cdot}75 \times 10^8$	$1{\cdot}70 \times 10^8$

[a] Acid hydrolysate
[b] Water extract
Counts at 0 h varies between $0{\cdot}87 \times 10^4$ to $0{\cdot}90 \times 10^4$ HSC/ml

Bioassay of β-exotoxin produced by B. thuringiensis var. thuringiensis grown on different test carriers and their combinations

The results presented in table 4 showed that various combinations of different test carriers, on which *B. thuringiensis* var. *thuringiensis* was grown for the production of crude *β*-exotoxin, affected the mortality of *Meloidogyne* sp. as compared to control, on the first day.

A critical examination revealed that the crude *β*-exotoxin obtained from the bacterial culture grown on acid hydrolysates of wheat bran + rice bran + water extract of farm yard manure resulted in complete mortality on the second day, by the acid hydrolysate of wheat bran + water extract of farm yard manure, acid hydrolysate of rice bran + water extract of farm yard manure, acid hydrolysate of wheat bran and lastly acid hydrolysate of rice bran were decreasingly effective. The crude *β*-exotoxin obtained from bacterial culture grown in the water extract of farm yard manure or on solid farm yard manure was less effective than other treatments on the first and second day (table 4).

In all the treatments the mortality on the seventh day due to *β*-exotoxin produced on different test carriers was very similar to that on the second day except in solid and water extract of farm yard manure, where the mortality increased from 20–23%. on the second day to 64–72% respectively on the seventh day (table 4).

Discussion

Prasad and Tilak (1972) and Srivastava (1973) have shown that *Meloidogyne* sp. can be effectively controlled in tomato and okra with *B. thuringiensis* var. *thuringi-*

Table 4. Per cent mortality of Meloidogyne spdue to *β*-exotoxin of *B. thuringiensis* var. *thuringiensis* produced on different test carriers and their combinations

Treatments	Per cent mortality					
	Control			*β*-exotoxin		
	1st day	2nd day	7th day	1st day	2nd day	7th day
Wheat bran[a]	Nil	8·00	17·32	44·00	75·32	95·00
Rice bran[a]	Nil	7·32	12·00	42·66	70·00	85·00
Wheat bran[a] + farm yard manure[b]	Nil	12·00	18·66	52·00	94·00	100·00
Rice bran[a] + farm yard manure[b]	Nil	11·32	17·32	51·32	90·00	100·00
Wheat bran[a] + rice bran[a] farm yard manure[b]	Nil	13·32	21·32	80·66	100·00	100·00
Farm yard manure[b]	Nil	Nil	4·00	4·66	20·00	64·00
Farm yard manure (solid)	Nil	12·00	34·00	6·00	23·20	72·00

[a] Acid hydrolysate
[b] water extract

ensis. In the present study it was observed that acid hydrolysates of wheat and rice brans gave maximum growth of the *B. thuringiensis* var. *thuringiensis*, because of easily available nutrients. Wheat and rice brans individually in solid form or as their water extract could not support the growth of the organism. This might be only due to nonavailability of the nutrients. However, these test carriers in different combinations with farm yard manure resulted in growth and sporulation of the organism, suggesting that farm yard manure provided nutrients for the growth and sporulation of the organism.

The solid or water extract of farm yard manure proved to be a good medium for the growth of *B. thuringiensis* var. *thuringiensis*. This was due to easily available sugars, minerals and nitrogen, etc. However, the *β*-exotoxin production was less by the bacterial culture grown on farm yard manure. The low production of *β*-exotoxin irrespective of the good growth of *B. thuringiensis* var. *thuringiensis* in farm yard manure could be attributed either to lack of sufficient amount of precursors, which may be essential for *β*-exotoxin production or adsorption of some of the *β*-exotoxin on farm yard manure particles which were not released during laboratory extraction of *β*-exotoxin with distilled water.

Assuming that per cent mortality was a direct reflection of amount of *β*-exotoxin produced, then the different combinations mentioned in results (table 4) were better for *β*-exotoxin production.

Consistent failure of *β*-exotoxin produced in water extract or on solid farm yard manure to cause mortality equal to the five treatments listed above might be due

to temporary inactivation of the β-exotoxin by some of the water soluble components, of farm yard manure or due to some modification in β-exotoxin which slowed down its action or the amount of β-exotoxin produced was relatively lower than the other media on account of which it required more time to cause killing. Further experiments are required to find out the actual reason for this.

Increased mortality on the seventh day due to β-exotoxin produced by *B. thuringiensis* var. *thuringiensis* grown on solid farm yard manure might be due to nematicidal activity of farm yard manure.

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