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Partial characterization of novel nematicidal toxins from *Bacillus cereus* Frankland and Frankland 1887 and their effect on root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood

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ABSTRACT: ***Bacillus cereus*** **isolated from the egg masses of root-knot nematode,** ***Meloidogyne incognita*** **from tomato rhizosphere was grown in casein-peptone soy meal peptone (CASO) broth for partial characterization of toxins from cell-free filtrates. Cell-free culture filtrates of *B. cereus* at 72 hours of growth and beyond, reduced egg hatching (90%) and caused 100 per cent mortality of juveniles in 4 hours of exposure, which coincided with the post-sporulation phase (72h) of the bacterial growth indicating profound toxicity during and after the sporulation phase. The native PAGE electrophoresis of the partially purified cell-free culture filtrates showed that 3 bands of ≈ 15, 40 and 60 kDa appeared in 72, 84 and 96 hours of growth, respectively, coinciding with post-sporulation phase of the bacterium.**

KEY WORDS: *Bacillus cereus, Meloidogyne incognita,* nematicidal toxins, partial characterization

INTRODUCTION

Bacillus cereus (IIHR BC1) was isolated from the egg masses of root-knot nematode, *Meloidogyne incognita* in tomato crop rhizosphere. The diseased egg masses exhibited very low percentage of hatching and the juveniles after hatching were dead. Microscopic examination of the diseased egg masses and juveniles did not show any deformation due to bacterial infection. These observations made us to suspect the production of nemato-toxins by *B. cereus*. Gokte and Swarup (1988) earlier reported that *Bacillus subtilis*, *B. pumilis*, *B. cereus* and two species of *Pseudomonas* were larvicidal on cyst and root-knot nematodes. Some of the gram-positive bacteria like *Streptococcus* sp., *Staphylococcus* sp., *Bacillus anthracis*, *Bacillus cereus* and *Clostridium* spp. produce toxins that act very similar to *Bt* (Lacy and Stevens, 1998). *In Vitro* studies were carried out on isolation, characterization of the toxins, and further evaluation of their effect on *M. incognita*.

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MATERIALS AND METHODS

Bacillus cereus (IIHR BC1) was isolated from the diseased egg masses of *Meloidogyne incognita* in tomato rhizosphere in 1999 using the standard isolation technique of surface sterilization-serial dilution and plating. The infected and discoloured egg masses were washed 2-3 times with sterile distilled water, surface-sterilized with 0.1per cent NaOCl for 2-3 seconds followed by washes with sterile distilled water to remove excess chlorine and sodium ions. The surface-sterilized egg masses were then homogenized in Eppendorf tubes (1.5 ml cap.) using a homogenizer. The homogenate was serially diluted with sterile distilled water and streaked on to the Petri-plates containing King's B medium. The moist-single-cell bacterial colonies were isolated separately and identified as per the taxonomic key (Bergey's Manual). *Bacillus cereus* was cultured on CASO (casein peptone soy meal peptone) broth for 7 days at 26 ± 1° C. The growth of the bacterium on CASO broth was recorded through UV-Vis scanning spectrophotometer (Genesis 5, Milton Roy Inc.) at 280 nm at 12 hour interval, while the filter-sterilized and cell-free culture filtrates were collected at 24, 48, 72, 96 and 168 hours of growth. The culture filtrates were bio-assayed against the freshly hatched second stage juveniles and uninfected egg masses of *M. incognita*.

Filter sterilized and cell-free culture filtrates obtained at 24, 48, 72, 96 and 168 hours were vacuum - concentrated and partially purified through sephadex G-25 column (2.5 cm x 30 cm). The fractions (at 280nm) were collected separately and tested for nematicidal activity. The concurrent fractions that exhibited nematicidal activity were then pooled. Aqueous suspension of juveniles was centrifuged at 500 rpm for 3 minutes and excess water pipetted out to have high number per 100μl. One hundred and fifty μl aliquots containing about 100 juveniles were placed in Eppendorf tubes (1.5ml cap.) to which 150μl of partially purified and vacuum-concentrated culture filtrates were added and incubated at 26 ± 1°C for 5 hours. The samples were placed in a watch glass at hourly interval and the numbers of active and dead juveniles were recorded under stereomicroscope. Complete mortality of nematodes was confirmed by placing the inactive nematodes in sterile water and observed for recovery. The fresh and healthy egg masses were collected from tomato roots with a fine-tip forceps, washed in sterile water, placed in sodium hypochlorite and the fresh, unhatched and healthy eggs were obtained (Hussey and Baker, 1973). One hundred μl aliquots containing about 100 eggs were placed in Eppendorf tubes (1.5ml cap.) to which 100μl of partially purified and vacuum-concentrated culture filtrates were added and incubated at 26 ± 1° C for 96 hours. The samples were placed in a watch glass at 24-hourly interval and the numbers of hatched juveniles were recorded under stereomicroscope.

The protein content of the retentate fraction was finally adjusted to a concentration of 1 to 2 mg/ml through freeze-drying and subjected to native electrophoresis. Native poly acryl amide gel electrophoresis (Native PAGE) with 10 per cent concentration of resolving gel (9.2 x 7.3 x 0.1 cm) and electrode buffer of 8.3 pH, respectively, was used. Coomassie Brilliant Blue 250 was used for staining the bands. The partially purified protein samples (~0.2 mg) from 24, 48, 72, 96 and 168 hours cultures were loaded along with medium range molecular marker (20-80 KDa) (Bangalore Genei) and the gel was run at 100 volts.

RESULTS AND DISCUSSION

The growth of *B. cereus* under batch culture conditions was recorded up to 96 hours. There was a positive correlation between absorbance and growth of the bacterium. The absorbance peak at 280 nm of cell cultures obtained from 24, 48, 72 and 96 hours cultures increased with increase in culture time (*i.e.* from 24 to 96 h). The growth of *B. cereus* (IIHR BC 1) followed a sigmoid curve with the exponential phase from 24 to 60 hours (Fig. 1).

Table 1. Mortality of *M. incognita* juveniles in cell free culture filtrates of *B. cereus* at different growth periods

Exposure time (hours)	Mortality (200 juveniles/assay) Age of cell-free culture filtrate of *B. cereus*					
	Control	24 h (X ± SEM)	48 h (X ± SEM)	72 h (X ± SEM)	96 h (X ± SEM)	168 h (X ± SEM)
1	0	20 ± 1.5 (10.00)	28 ± 2.0 (14.00)	68 ± 3.5 (34.00)	84 ± 3.0 (42.00)	108 ± 3.5 (54.00)
2	0	36 ± 2.0 (18.00)	40 ± 3.0 (20.00)	94 ± 5.0 (47.00)	118 ± 6.0 (59.00)	166 ± 5.0 (83.00)
3	0	40 ± 2.5 (20.00)	44 ± 3.0 (22.00)	110 ± 5.0 (55.00)	146 ± 6.5 (73.00)	200 ± 0.0 (100.00)
4	0	60 ± 3.5 (30.00)	62 ± 3.0 (31.00)	136 ± 5.5 (68.00)	176 ± 7.5 (88.00)	-
5	0	68 ± 3.5 (34.00)	86 ± 4.5 (43.00)	168 ± 5.5 (84.00)	190 ± 7.0 (95.00)	-

Values in parentheses indicate per cent juvenile mortality.

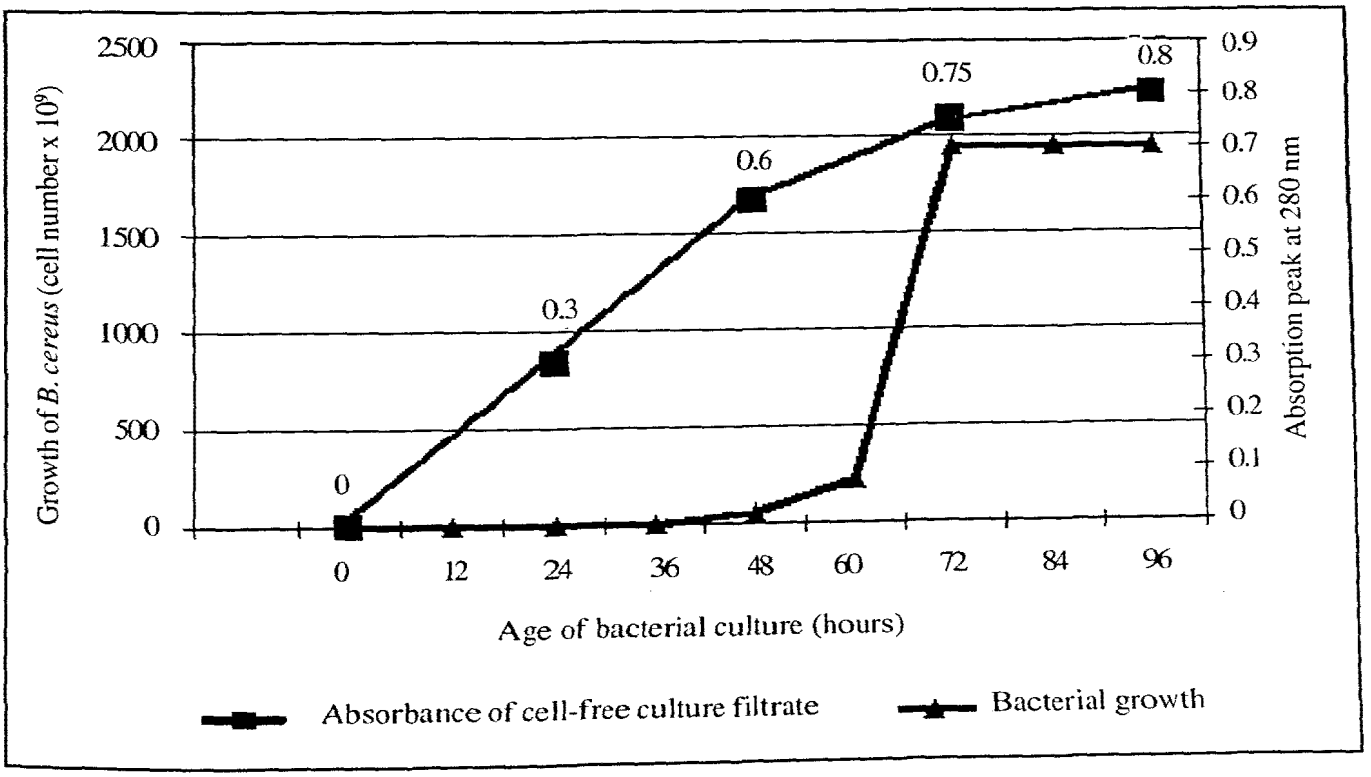

Figure 1. Absorbance peaks of *B. cereus* cells during growth phase and cell-free culture filtrates

Partially purified culture filtrates at different growth stages caused mortality to juveniles and inhibited egg hatching with increase in exposure time compared to the bacterium-free CASO broth. Mortality of juveniles and inhibition of egg hatching were not significant in partially purified cell-free culture filtrates up to 72 hours of growth (Table 1 & 2). However, culture filtrates at post 72 hours of growth, reduced egg hatching up to 90 per cent and caused 100 per cent mortality of juveniles in 4 hours of exposure. Maximum mortality of juveniles and inhibition of hatching coincided with the post-sporulation phase (72 h) of the bacterium indicating profound toxicity during and after the sporulation phase.

Table 2. Effect of cell free culture filtrates of *B. cereus* on egg hatching of *M. incognita*

Exposure time (hours)	Mortality (100 eggmasses/assay) Age of cell-free culture filtrate of *B. cereus*					
	Control ($\bar{X} \pm$ SEM)	24 h ($\bar{X} \pm$ SEM)	48 h ($\bar{X} \pm$ SEM)	72 h ($\bar{X} \pm$ SEM)	96 h ($\bar{X} \pm$ SEM)	168 h ($\bar{X} \pm$ SEM)
24	196 ± 5.5	178 ± 7.5 (9.00)	166 ± 8.0 (15.00)	160 ± 6.0 (18.00)	140 ± 2.0 (29.00)	22 ± 2.0 (89.00)
48	188 ± 6.0	188 ± 8.0 (0.00)	180 ± 9.5 (4.00)	154 ± 5.5 (18.00)	118 ± 1.5 (38.00)	46 ± 2.5 (76.00)
72	260 ± 6.5	212 ± 6.0 (18.00)	184 ± 6.5 (29.00)	134 ± 8.5 (48.00)	102 ± 4.0 (61.00)	32 ± 1.5 (88.00)
96	218 ± 9.0	188 ± 6.5 (9.00)	140 ± 5.0 (36.00)	122 ± 5.5 (44.00)	98 ± 4.5 (55.00)	22 ± 1.0 (90.00)

Values in parentheses indicate per cent inhibition of hatching.

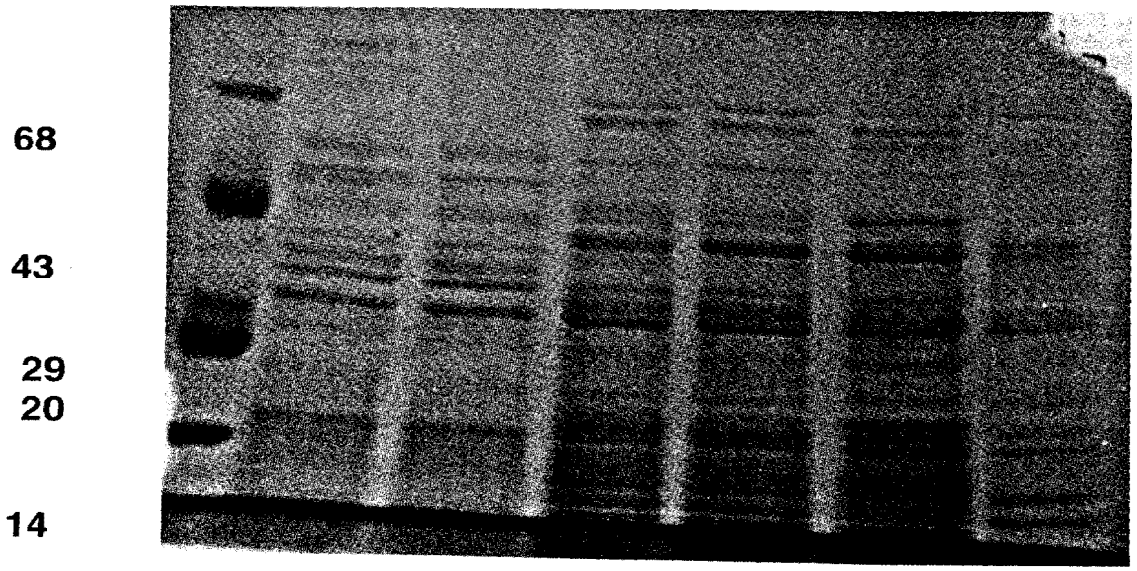

Fig. 2. Native PAGE electrophoresis of partially purified toxins from cell-free culture filtrates of *Bacillus cereus*

Analysis of the native PAGE of culture filtrates at different durations revealed that three major bands of ca.15, 40 and 60 KDa appeared prominently from 72 hours of cell free and partially purified cultures. Three bands of ca. KDa appeared in 72, 84 and 96 hours of growth cultures coinciding with post-sporulation phase of the bacterium and maximum juvenile mortality phase (Fig. 2).

Many bacterial toxins, including insecticidal toxin of *Bacillus thuringiensis*, are known to be composed of multiple sub-units (Hofte and Whitely, 1989). Although, *Bacillus subtilis*, *B. pumilis*, *B. cereus* and 2 species of *Pseudomonas* were reported to be nematicidal (Gokte and Swarup, 1988), the isolation and characterization of the toxins were not undertaken. In the present study, it is clear that cell-free filtrates of 72-hour-old cultures and above, exhibited toxic activity, which could be due to one protein or a combination of protein sub-units. The significance of these observations under the study, is that the novel nematicidal toxins of proteinacious nature from soil and rhizoplane bacteria, *viz. Bacillus* and *Pseudomonas* spp. can be explored and exploited for the management of nematode pests.

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