EFFECTS OF CRUDE ANTIBIOTIC OF *BACILLUS SUBTILIS* ON HATCHING OF EGGS AND MORTALITY OF JUVENILES OF *MELOIDOGYNE INCOGNITA*

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Summary. The endospore-forming rhizobacterium *Bacillus subtilis* produces more than two dozen lipopeptide antibiotics, hydrolytic enzymes and other secondary metabolites. The biocontrol activity of *Bacillus* strains against multiple plant pathogens has been widely documented. Three families of *Bacillus* lipopeptides (surfactins, iturins and fengycins) are the most studied for their antagonistic activity against a wide range of phytopathogens, including bacteria, fungi and nematodes. The root-knot nematode, *Meloidogyne incognita*, is one of the most damaging pathogens, attacking a wide range of crops. Therefore, six antagonistic endophytic strains of *B. subtilis viz.,* Bs N 1*,* Bs N 3, Bs N 4, Bs N 7, Bs 5 and Bs N 11, were isolated from noni plants and tested for their nematicidal activity against *M. incognit*a. The strain Bbv 57, isolated from banana and known to have nematicidal activity, was included in the study as a control. The genomic DNA of the *Bacillus* strains was isolated and amplified by PCR to identify antibiotic genes. Biosynthetic gene specific primers amplified a 440 bp fragment of *surfactin* gene from BsN 3, Bs 5 and Bbv 57 and a 648 bp fragment of *iturin* gene from Bs 5 and Bbv 57. Bs 5, with high surfactin and iturin activity, suppressed hatching of eggs and killed second stage juveniles of the nematode under *in vitro* conditions.

Key words: Antibiotics, control, *iturin* gene, root-knot nematode, *surfactin* gene.

The root-knot nematode, *Meloidogyne incognita* (Kofoid *et* White) Chitw., is one of the most damaging of agricultural pests. Kavitha *et al*., 2011 reported that yield loss caused by root knot nematode in noni was 46%. Opportunistic or pathogenic fungi and bacteria that colonize nematode-infected tissues may exacerbate the symptoms and the disease complex may result in death of the plant (Nelson, 2005). Since chemical control is becoming more and more expensive and its use is undesirable due to environmental concerns, biological control of plant-parasitic nematodes is being suggested as an economical and eco-friendly control option.

Bacillus species are outstanding biocontrol agents that are used to manage root-knot nematodes as they show effective root colonization, multiple modes of action and promising ability to sporulate (Kloepper *et al.*, 2004). They live as endophytes within the plant and benefit the host plant by preventing pathogenic organisms from colonizing them. An important characteristic of this bacterium genus is its ability to produce endospores when environmental conditions are stressful. Lytic enzymes *viz.,* chitinase and glucanase are known to be produced by *Bacillus* (Csuzi, 1978). Cyclic lipopeptides of the surfactin, iturin and fengycin families are important metabolites produced by *Bacillus* species (Gray *et al*., 2006). The enzymes and antibiotics produced by *B. subtilis* (Ehrenberg) Cohn have been reported to have antagonistic effects on pathogenic microorganisms (Doherty and Preece, 1988). They impart successful biocontrol activity by direct suppression of phytopathogens and by further protecting the host plant through stimulating systemic resistance. Therefore, an investigation was undertaken to explore the nematicidal activity of culture filtrates of the promising biocontrol agent *B. subtilis* and to elucidate the underlying mechanisms responsible for their antagonistic activity towards root knot nematodes.

MATERIALS AND METHODS

Isolation of endophytic *B. subtilis* strains

Six antagonistic strains of *B. subtilis viz.*, Bs N 1*,* Bs N 3, Bs N 4, Bs N 7, Bs 5 and Bs N 11, were isolated from noni (*Morinda citrifolia* L.) plants collected in the medicinal plants block of the Tamil Nadu Agricultural University, Coimbatore, India (Hassan *et al*., 2010). Healthy tissues of leaves and stems of noni plants were put in a beaker, soaked in distilled water and then drained. Tissues were rinsed in 70% ethanol for 30 seconds and then sterilized with a 0.1% $HgCl_2$ solution for 3 minutes. The tissues were then washed ten times with sterile water (Gagne *et al*., 1987). Surface-disinfected tissues were aseptically macerated in a homogenizer. The macerated tissues were diluted ten times by adding nine volumes of sterile distilled water. Serial dilutions were made up to 10^{-6} by taking 1 ml of well-shaken suspension and 9 ml water in tubes. One hundred µl samples from the dilutions were spread on plates of NA medium in sterile Petri dishes. The *B. subtilis* strain Bbv 57, reported to be effective against root knot nematode, was obtained from the Centre for Plant Protection and used in the study for comparison.

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Detection of antibiotic genes of *Bacillus* isolate

To be sure that the culture filtrates of our strains of *B. subtilis* contained crude antibiotics, the genomic DNA from the *Bacillus* strains was isolated using the cetyl trimethyl ammonium bromide (CTAB) method described by Knapp and Chandlee (1996), with slight modifications (Melody, 1997).

PCR amplification of antibiotic genes

Surfactin. The forward primer SUR3F (5' ACAGTATGGAGGCATGGTC 3') and reverse primer SUR3R (5' TTCCGCCACTTTTTCAGTTT 3') were used for amplification of *surfactin* gene (440 bp) (Ramarathnam, 2007). The 40 µl PCR reaction mixture contained DNA template 50 ng, 1xTaq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primer, 1.5 mM $MgCl_2$ and 2U of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) using the following conditions: initial denaturation at 94 °C for 3 min, 40 cycles consisting of 94 °C for 1 min (denaturation), 57 °C for 1 min (annealing), 72 °C for 1 min (primer extension) and final extension at 72 °C for 10 min.

Iturin A. The forward primer ITUD1F (5' GATGCGATCTCCTTGGATGT 3') and reverse primer ITUD1R (5' ATCGTCATGTGCTGCTTGAG 3') were used for amplification of *iturin A* gene (648 bp) (Ramarathnam, 2007). The 20 µl mixture contained approximately 50 ng of total DNA, 5 mM of each dNTPs, 20 pmol each of the forward and reverse primers and 0.5 U of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, Germany) using the conditions: initial denaturation at 94 °C for 3 min, 40 cycles consisting of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), 72 °C for 1 min (primer extension) and final extension at 72 °C for 10 min.

Agarose gel electrophoresis and Gel documentation

Agarose gel electrophoresis was performed based on the method given by Sambrook *et al*. (1989) to check the quality of DNA and also to separate the products amplified through the polymerase chain reaction. After separation on 1.5% agarose gel at 50 UV, the PCR products were stained with ethidium bromide (0.5 µg/ml), photographed using Alpha imager TM1200 and analyzed using a gel documentation system (Alpha Innotech Corporation, San Leandro, California).

Extraction of crude antibiotic from *B. subtilis*

The bacterial strain Bs 5 was selected for testing its nematicidal activity. Besides showing surfactin and iturin activity (see results), this strain had also shown activity against the fungal pathogens of noni *Alternaria* sp and *Colletotrichum* sp. (Chandra Sekar, unpublished data).

The bacterial strain was grown at 28 ± 2 °C for 5 days in pigment production broth containing peptone (20 g/l), glycerol (20 g/l), NaCl (5 g/l) and KNO_3 (1 g/l) in 1000 ml of distilled water at pH 7.2. After incubation, the bacterial culture was centrifuged at 7840 g, the supernatant was adjusted to pH 2.0 with concentrated HCl and the crude antibiotic extracted with an equal volume of different solvents (chloroform, ethyl acetate and butanol) by shaking at 200 rpm for approximately 8 hrs in an orbital shaker. The organic solvent layer was evaporated in a water bath maintained at 100 °C. After evaporation, the precipitate was re-suspended in chloroform: methanol (1:1) and the supernatant removed by centrifugation. The crude antibiotic solution was concentrated in a vacuum flask evaporator. The residues were collected in Eppendorf tubes and stored at 4 °C. This crude antibiotic was used for nematicidal tests, for which different concentrations were prepared by dilution with distilled water.

***In vitro* experiments**

Two *in vitro* bioassays were conducted simultaneously to assess the nematicidal activity of the crude antibiotic of the *B. subtilis* strain Bs 5.

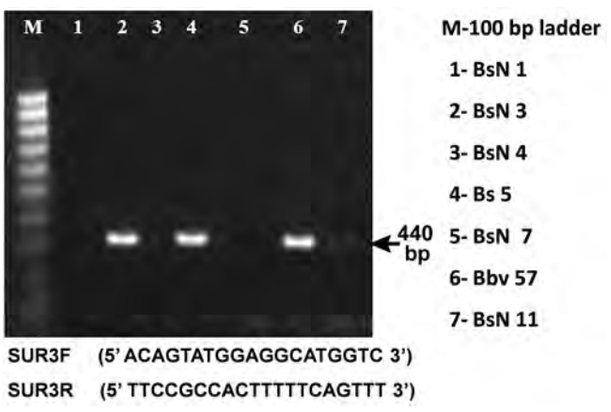

Fig. 1. PCR amplification of the antibiotic biosynthetic gene *Surfactin* of *Bacillus* isolates.

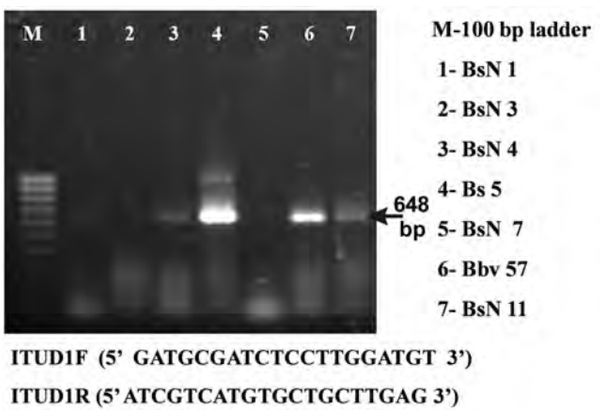

Fig. 2. PCR amplification of the antibiotic biosynthetic gene *Iturin* A of *Bacillus* isolates.

Hatching test. Eggs masses of *M. incognita* were obtained from the infected roots of noni plants. Sinlge egg masses, containing an average of 300-350 eggs, were placed in Syracuse dishes and treated with 2 ml of crude antibiotic of the *B. subtilis* strain Bs 5, which has *surfactin* and *iturin* genes, at concentrations of 100, 50 and 25% and kept at 28 ± 1 °C for different exposure times. Syracuse dishes without antibiotic and treated instead with distilled water served as control. Each treatment was replicated three times. The numbers of juveniles emerged from the eggs were counted after 24, 48 and 72 h. The experiment was repeated.

Mortality test. Two ml of crude antibiotic at concentrations of 100, 50 and 25% were placed in separate Syracuse dishes. Egg masses of root knot nematode were collected from the infected roots of noni plants and incubated in water for 3 days to allow eggs to hatch. After 3 days, 100 second stage juveniles were added to the Syracuse dishes and incubated at 28 ± 1 °C. Juveniles placed in dishes containing distilled water served as control. There were three replicates of each treatment. Observations were recorded on the mortality of juveniles at 24, 48 and 72 h intervals using a stereoscope. Thereafter, juveniles treated with the crude antibiotic were transferred to distilled water and observed again after 24 hrs to confirm their mortality. This test was also repeated.

Statistical analysis

As the data from the two experiments per test were similar, they were pooled, statistically analyzed and critical differences determined as per the method given by Gomez and Gomez, (1984). Egg hatching data were square root transformed and mortality data were arcsine transformed before analysis.

RESULTS

PCR amplification of antibiotic genes

The PCR amplified products, after separation in agarose gel electrophoresis followed by gel documentation, revealed the presence of *surfactin* and *iturin* genes in the *B. subtilis* strains. Biosynthetic gene specific primers SUR3F and SUR3R amplified a 440 bp fragment of *surfactin* gene for BsN 3, Bs 5 and Bbv 57 (Fig. 1). Iturin specific primers amplified a fragment of 648 bp of *iturin* gene for BsN 4, Bs 5, Bbv 57 and BsN 11 (Fig. 2).

Hatching test

The strain Bs 5, with *surfactin* and *iturin* genes, was found promising in reducing hatching of *M. incognita* eggs to a varying degree. The crude antibiotic extracted from the strain Bs 5 exerted lethal effects on eggs and juveniles of the root knot nematode. Egg hatching was hindered and only a few juveniles emerged. Juveniles inside the eggs were dead, thereby proving the ovicidal effect of the crude antibiotic. A significant reduction in egg hatching (an average of only 16.3 eggs hatched) was observed at 100% concentration after 72 h of exposure,

Table I. Effect of crude antibiotic of *B. subtilis* strain Bs 5 on hatching of eggs and mortality of second stage juveniles of *Meloidogyne incognita* at different exposure times.

Crude antibiotic concentration (%)	Exposure time (hrs)					
	No. of eggs hatched/egg mass			No. of juveniles dead /100 juveniles *		
	24	48	72	24	48	72
100	7.9 (2.78)	12.6 (1.61)	16.3 (4.02)	60.6 (1.78)	70.2 (1.84)	92.6 (1.96)
50	12.7 (3.37)	29.0 (5.45)	36.5 (6.03)	38.2 (1.88)	48.1 (1.68)	62.4 (1.78)
25	34.1 (5.83)	38.2 (6.18)	42.4 (6.51)	30.1 (1.47)	40.7 (1.60)	46.5 (1.66)
Control (Distilled water)	75.5 (8.78)	167.2 (12.93)	286.5 (6.92)	0.0 (0.28)	0.0 (0.28)	4.0 (0.64)
CD (0.05)	0.37	0.27	0.18	0.04	0.03	0.55

Figures in parenthesis are square root transformed values

*Figures in parenthesis are arcsine transformed values

Pooled analysis of data collected from two sets of experiments

compared to 86.5 eggs in the control (Table I). In both experiments, the lethal effect of the antibiotic resulted in complete paralysis and death of the juveniles inside the eggs after 72 h of exposure.

Mortality test

The crude antibiotic also had a lethal effect on the juveniles of the nematode as shown by the reduced movement and greater death rate than in the control. The reduction in the movement was irreversible and the death of the juveniles was confirmed when they were transferred to distilled water for 24 hrs. A gradual increase in juvenile mortality was observed with the increase in concentration of the crude antibiotic and with the increase in the exposure time. The greatest juvenile mortality (an average of 92.3 J_2) was recorded in the 100% concentration of the crude antibiotic after 72 h of exposure (Table I).

DISCUSSION

The biocontrol activity of *Bacillus* strains against multiple plant pathogens is associated with the ability to produce lipopeptide antibiotics that exhibit a wide spectrum of antinemic activity. Production of surfactin by *Bacillus* is a characteristic that supports their persistence under extreme conditions (Kloepper *et al.*, 2004). Genes encoding surfactin synthesis are common to numerous antagonistic *Bacillus* spp. used as commercial biopesticides (Joshi and Gardener, 2006). In our experiments, the culture filtrate of *B. subtilis* effectively inhibited hatching of eggs and killed juveniles of the nematode. Siddiqui *et al*. (2000) obtained similar results on the mortality of *M. javanica* using ethyl acetate and hexane fractions at different concentrations. Hence, *B. subtilis* could be a valuable candidate for biological control of root knot nematodes. Therefore, pot culture and field experiments are necessary to validate these results and to obtain insights on the factors that influence root colonization and thereby persistence in the rhizosphere and the key role played in the beneficial interaction of *Bacillus* species with plants by stimulating host defense mechanisms against phytopathogens, including nematodes.

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