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Evaluation of Culture Filtrate of an Entomopathogenic Bacterium for Nematicidal Properties against Root-Knot Nematode, *Meloidogyne incognita*

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Abstract

Bacterial culture, its culture filtrate and organic extract were tested *in vitro* against infective juveniles (IJ) of *Meloidogyne incognita*. Nematicidal activity was observed in 24-96 h exposure time with bacterial culture, cell free culture filtrate and organic crude extract at various concentrations. Mortality rate increased with concentration and exposure time. Bacterial culture filtrate using TSB, LB and NB media recorded 50%, 36.6% and 13.3% mortality respectively in 96 h exposure time at a concentration of 150 µl/ml. Bacterial culture filtrate of TSB + Fructose exhibited nematicidal activity of 16.6% and 80% at a concentration of 150 µl/ml in 24 and 96 h exposure time respectively which was higher than other media used. Organic crude extract obtained using TSB + Fructose media recorded a mortality of 70% and 100% at 2.5µg/ml and 5µg/ml concentration respectively in 24 h exposure period.

Key words: *Meloidogyne incognita*, nematicidal activity, root-knot nematodes, *Steinernema*

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are one of the most economically important pests causing severe damages to a wide variety of crops. Even though over 90 species of the genus *Meloidogyne* have been described worldwide (Moens et al., 2009; Sikora and Fernandez, 2005), only *M. incognita*, *M. javanica*, and *M. arenaria* are of agronomic importance, being responsible for at least 90 % of all damage caused by root-knot nematodes (Castagnone-Sereno, 2002). Cultural practices like crop rotations, resistant varieties, and/or applying nematicidal agents are not adequate (Roberts, 1992). Natural compounds with nematicidal activities (NAs) are of significant economical importance in agriculture. The use of biological products is becoming appealing because of the growing problem of environmental pollution arising from the use of persistent pesticides and also after prolonged use the efficiency of chemical nematicides are found decreasing.

Entomopathogenic nematodes (EPN) and their symbiotic bacteria play an important role in suppression of insect pest population in soil (Chen et al., 1996). EPN families, steinernematidae and heterorhabditidae are symbiotically associated with bacteria *Xenorhabdus* and *Photorhabdus*, respectively. The bacteria are carried by the third stage infective juvenile (IJ3) of the nematode, which infects the insect host by entering through natural openings. Several of these bacteria produce bioactive compounds possessing antimicrobial and insecticidal activity in *in vitro* cultures. Nutrients and culture conditions were found to influence the production of these antimicrobial compounds of EPN bacteria (Nishanth et al., 2012). An entomopathogenic nematode belonging to the genus

Steinernema was isolated from soil collected from Namakkal district (Tamil Nadu).The nematode was associated with a novel bacterium and the present study is focused on the screening of nematicidal activity of entomopathogenic bacterium against root-knot nematode, *M. incognita* in *in vitro* condition which may help to prevent the root diseases in econo-medicinal plants.

Materials and Methods

Bacteria and nematodes

Bacteria: The entomopathogenic bacteria used in this study was isolated from EPN belonging to the genus *Steinernema*, isolated from soil collected from Namakkal district (Tamil Nadu) and maintained at CTCRI. The technique described by Park (1999) was used to isolate bacteria from the nematode. The bacterial culture was maintained in nutrient agar by sub culturing.

Root-knot nematode: Egg masses of *M. incognita* were picked up from the root-knots using dissecting needle from infected roots of tomato plant and placed in watch glass containing sterile distilled water. The second stage juveniles (J2s) emerging from the egg masses were inoculated onto ornamental plant coleus and maintained in earthen pots. The root knots from this plant were used for all the experiments.

Fermentation

A loopful of freshly cultured bacteria was inoculated into 100 ml broth and incubated in a shaking incubator at 30°C at 150 rpm for 24h. This 24h seed culture is used to start the fermentation in 1L conical flask containing 400 ml media under same culture conditions.

Extraction of organic fraction

Fermented culture (1L) was subjected to centrifugation at 10,000 rpm for 12 minutes. The supernatant was transferred to a separation funnel to which equal volume of ethyl acetate was added. The funnel was strongly agitated and kept for half an hour for the separation of the organic and aqueous phases. This process was repeated twice. The ethyl acetate extracts were combined and concentrated in a rotary evaporator at 40°C and dissolved in 1 ml methanol and stored at -20°C.

Nematicidal activity

Effect of bacterial culture on nematode mortality

One day old bacterial cultures from three basal media (TSB, LB and NB) were tested for nematicidal activity. Nematicidal activity of the bacterial culture was determined by adding freshly hatched nematode juveniles into cavity blocks. One ml of bacterial culture (undiluted and serially diluted) was taken in 3cm diameter cavity block to which ten J2s were added and observed for mortality in every 24h up to 96h of exposure time using stereo microscope (ZEISS KL 1500 LCD). Nematodes were considered dead if they remain immobile on probing with a fine needle (Cayrol et al., 1989). Immobile J2s were transferred to sterile distilled water and observed for 48h to check their potential for revival. Cavity blocks containing same volume of sterile media as in the sample and water were used as control.

Effect of basal media on nematicidal activity

Several basal media (HiMedia) like tryptone soya broth (TSB), luria broth (LB) and nutrient broth (NB) were assessed to identify the preferred medium for the nematicidal activity. To determine the effect of carbon sources on nematicidal activity different carbon sources such as glucose, fructose, maltose and sucrose were added to the preferred basal medium (TSB). The amount of carbon compounds added to the medium was adjusted to give the total carbon concentration equal to 1%.

Effect of fermentation period on nematicidal activity

To determine the effect of fermentation period the fermentation was carried out for 24, 48, 72, 96 and 120h and nematicidal activity was carried out at the end of each fermentation period.

Effect of bacterial culture filtrate on nematode mortality

To determine the effect of bacterial culture filtrate on juveniles, 150 µl of the filtrate was transferred into cavity block containing 850 µl of sterile distilled water and to that 10 J2s were added. Cavity blocks containing same volume of water and sterile media was used as control. Mortality was checked as described earlier.

Effect of organic extract on nematode mortality

One ml of sterile distilled water was taken in 3 cm diameter cavity block to which organic extract was added. Nematicidal activity was studied at three different concentrations (1.25, 2.5 and 5 µg/ml). Ten J2s were transferred to each cavity block. Cavity blocks containing 10 µl of methanol in 990 µl sterile distilled water and sterile distilled water were used as control. Mortality was checked as described above.

All treatments were replicated three times and percentage mortality was calculated according to Abbott's formula (Abbott, 1925).

Abbott's formula

$$\text{Mortality}(\%) = \frac{m-n}{100-n} X100$$

Whereas: m = percentage mortality in treated sample and n = control

Statistical analysis

Statistical analysis software (SPSS/version17.0 software) was used to evaluate nematicidal activity. Overall differences among means were tested using one-way analyses of variance (ANOVA). Duncan's test was used to test significant differences among individual means if significant overall treatment effects were found at $P < 0.05$. Results are reported as mean ± standard error. Data were subjected to probit analysis to calculate LD50.

Results and Discussion

Effect of bacterial culture on nematode mortality

Bacterial culture from all the three basal media showed 100 % mortality in 24 h exposure time (Table 1). This result was significant when compared with control. At 10^{-1} dilution, culture showed low mortality when compared to undiluted bacterial culture. Nematicidal activity was found increasing with the concentration of bacterial cell in culture broth. No mortality was observed in control. These results are in line with those reported by Racke and Sikora (1992) who found that the antagonistic activity of *Agrobacterium radiobacter* or *Bacillus sphericus* against *Globodera pallida* were directly correlated with the number of colony forming units. Huangin et al. (2010) found out significant reduction in gall formation and egg mass formation with increase in the bacterial concentration.

Effect of media and fermentation period on nematicidal activity

Among the three basal media tested, the highest nematicidal activity of 100 % mortality was obtained from TSB in 24 h fermentation period in 24 h exposure time when compared to other basal media (Table 2). Similar nematicidal activity of bacterial culture against freshly emerged J2s of *M. incognita* within 24-48 h exposure time was reported by Hadad et al. (2010).

Table 1. Effect of bacterial culture on nematode mortality in 24 h exposure time

Media	Mortality (%)
TSB	100
LB	100
NB	100
TSB 10^{-1} dilution	10
LB10^{-1} dilution	6
NB10^{-1} dilution	3
Control (water)	0

Effect of bacterial culture filtrate on nematode mortality

Bacterial culture filtrate of TSB + Fructose exhibited maximum nematicidal activity of 16.6 % and 80 % at a concentration of 150 µl/ml in 24 and 96 h exposure time respectively, which was higher than other media used (Fig 1). TSB + Maltose, TSB + Glucose and TSB + Sucrose exhibited nematicidal activity of 13.3 %, 13.3 % and 6.6 %, respectively at 24 h exposure time and 73.3 %, 66.6 % and 43.3%, respectively at 96 h exposure time. No mortality was observed in control.

The present study demonstrated that nutritional factors have significant impact on the antagonistic activity of the

Table 2. Effect of fermentation time and basal media for nematicidal activity in 24h exposure time at a concentration of 5 µg/ml

Basal media	Fermentation period (h)				
	24	48	72	96	120
TSB	10c	3.33b	0a	0a	0a
LB	1.66b	0a	0a	0a	0a
NB	0a	0a	0a	0a	0a

* Means in the same column sharing a common letter are not significantly different according to Duncan's test at $P < 0.05$

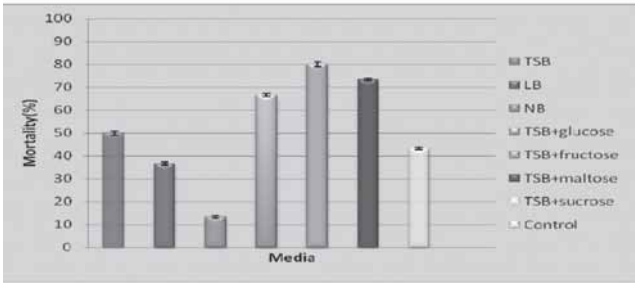

Fig. 1. Effect of cell free culture filtrate on nematicidal activity at 150 µl/ml concentration in 96 h exposure time

antagonist against the pathogen. Culture filtrate of all the media exhibited significant nematicidal activity. Nematicidal activity varied with the media provided for fermentation. Among the basic media used for fermentation culture filtrate using TSB showed maximum nematicidal activity, which increased with the supplement of different carbon sources. TSB with fructose exhibited maximum nematicidal activity. The impact of nutritional factors (different carbon and nitrogen sources) on bioactivity of the metabolites was also reported by Zeinat (2010). Nematicidal activity of culture filtrate was also reported by Afzal et al. (2013).

Effect of organic extract on nematode mortality

When the basal media was modified by adding extra carbon source, the activity increased. At 2.5µg/ml concentration in 24 h exposure time TSB + Glucose, TSB + Maltose and TSB + Fructose showed 23.33 %, 66.66 % and 70.00 % mortality, respectively. The mortality increased with increase in concentration (Fig. 2) and exposure time (Table 3). The LD_{50} value of TSB + Maltose, TSB + Glucose and TSB + Fructose were calculated as 1.0786, 1.552, and 0.925 µg/ml, respectively in 96 h exposure time. Sadrati et al. (2013) used ethyl acetate to extract crude organic compound for testing antimicrobial activity and got significant activity against bacteria, fungi and yeast. In this study also ethyl acetate extract showed good nematicidal activity against *M. incognita*.

From the present work it is possible to conclude that these microorganisms could be promising source of bioactive compounds, and warrant further study. The information obtained is considered fundamental and useful for the development of nematicidal compounds. Result showed that the organic extracts from this novel entomopathogenic bacterium have potential to be developed as nematicides, but the use of this against the root knot nematodes needs further study before being used as an alternative to chemical nematicide.

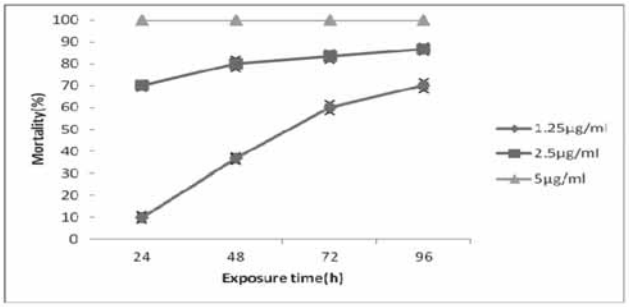

Fig. 2. Nematicidal activity of organic extract of TSB + Fructose at 1.25 µg/ml, 2.5 µg/ml and 5 µg/ml concentrations in 24 h to 96 h of exposure time. Percent mortality increased with the concentration and exposure time

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Table 3. Effect of media composition and exposure time on nematicidal activity of organic crude at a concentration of 1.25 µg/ml

Media	Exposure time (h)			
	24	48	72	96
TSB	1.33 ± .33b	2.33 ± .33b	3.33 ± .33b	4.33 ± .33bc
TSB + Glucose	0a	0a	2.66 ± .88b	3.33 ± 1.20b
TSB + Sucrose	0a	0a	0a	0a
TSB + Maltose	0a	2.66 ± 1.201b	4 ± 1.52bc	5.66 ± 1.76bc
TSB + Fructose	1 ± .57b	3.66 ± .667b	6 ± 1c	7 ± 1.00c
Control Water	0a	0a	0a	0a

Means in the same column sharing a common letter are not significantly different according to Duncan's test at $P < 0.05$

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