



Research Article

IN VITRO EVALUATION OF DIFFERENT STRAINS OF *BACILLUS* SPP. AGAINST ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA*

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ABSTRACT

Rhizospheric bacteria, *Bacillus* species are widely evaluated as a biological control agent against root-knot nematode (*Meloidogyne incognita*), causing infection in various crops. There are various strains that were found to be effective against root-knot nematode. The culture filtrate of these bacterial strains contains secondary metabolites that showed certain nematicidal properties. An *in vitro* experiment was conducted to evaluate the inhibitory properties of a few bacterial culture filtrates on egg hatching of *M. incognita*. The inhibitory effect varied according to rhizobacterial strains, different concentration levels and exposure time. After 24h of incubation, very little egg hatching was recorded at all the concentrations. Reducing the concentration of CFs showed a positive effect on egg hatching in case of all the rhizobacterial strains. Among the five rhizobacterial strains, the maximum hatching inhibition was observed in the case of *Bacillus aryabhathi* (KMT 4), which was around 19.9%, followed by *Gluconacetobacter diazotrophicus*, having 22.1% hatching at S/2 concentration. All other rhizobacterial strains significantly reduced egg hatching at all the concentrations as compared to the untreated check (67.5%). The minimum hatching was observed at a higher concentration of S/2 and it gradually increased with an increase in the dilutions, irrespective of the exposure period.

Keywords: Rhizospheric bacteria, *Meloidogyne incognita*, *Bacillus aryabhathi*, *Gluconacetobacter diazotrophicus*.

INTRODUCTION

Plant parasitic nematodes (PPNs), especially root-knot nematodes (RKNs) of the genus *Meloidogyne*, are a major constraint on global food security. These microscopic roundworms cause an estimated annual loss of over USD 80 billion to agriculture worldwide (Nicol *et al.*, 2011; Abd-Elgawad, 2024). Among them, *Meloidogyne incognita* stands out as one of the most economically significant and destructive species, known to infect more than 5,500 plant species globally (Jones *et al.*, 2013; Song *et al.*, 2023). The life cycle of *M. incognita* progresses from the egg stage to infective juveniles and finally to adults, with the juveniles being the primary cause of damage by penetrating plant roots. As an obligate plant parasite, these microscopic organisms establish specialized feeding structures called giant cells within plant root tissue. This process diverts essential plant resources and energy toward nematode

nutrition and leads to the formation of galls in the vascular bundles of the plants. The physical damage and impaired function of the roots lead to reduced nutrient and water uptake, resulting in visible above-ground symptoms such as stunting, yellowing, wilting, and substantial yield losses. The challenge of managing *M. incognita* is further compounded by its high fecundity rate, short generation time, and the ability of its eggs to survive under adverse conditions, allowing multiple generations to infest a single crop season.

Both fungi and bacteria are effective antagonists of nematodes. Bacterial antagonists classified as epiphytic, endophytic, or endoparasitic exert control through antibiosis, competition, parasitism, and plant growth promotion (Abd-Elgawad and Kabeil, 2012; Backer *et al.*, 2018). Bacteria associated with plants and soil exhibit diverse suppressive activities against plant-parasitic

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nematodes by producing toxins, antibiotics, and enzymes that interfere with nematode development and infectivity (Khan *et al.*, 2004). Among these, rhizobacteria such as *Pseudomonas* spp. and *Serratia marcescens* have been widely reported as bio-control agents against *Meloidogyne incognita*. Their nematicidal potential arises both from direct inhibition of nematode infectivity factors and from indirect growth-promoting effects on host plants (Siddiqui and Akhtar, 2009a, Siddiqui and Akhtar, 2009b, Khanna *et al.*, 2019). For example, *S. marcescens*, a known chitinase producer, inhibits the growth of phytopathogenic and saprophytic fungi, thereby indirectly suppressing nematodes (Okay *et al.*, 2013).

Similarly, *Pseudomonas fluorescens* and *Bacillus subtilis* have been successfully applied as seedling root tips or soil drenches, reducing gall formation and nematode populations while improving host plant growth (Sonkar *et al.*, 2018; Nyodu and Das, 2020). *Bacillus* spp. stand out as promising bio-control agents due to their strong root colonization, ability to sporulate, and multiple modes of action. They produce lytic enzymes and cyclic lipopeptides (surfactin, iturin, fengycin) with nematicidal and anti-fungal activities, along with ISR-inducing effects (Kloepper *et al.*, 2004; Gray *et al.*, 2006). Culture filtrates of *B. subtilis* have shown significant antagonistic effects on root-knot nematodes by direct suppression and stimulation of host defenses.

MATERIALS AND METHODS

Nematicidal activity of rhizobacterial strains viz., *Bacillus aryabhattii* KMT4, *B. cereus* KMT5, *B. atitudinis* KMS6, *B. megaterium* KMT8 and *Gluconacetobacter diazotrophicus* 35-47 was evaluated *in vitro* against egg hatching of *M. incognita* at various dilutions viz., S/2, S/4 and S/6. The inoculum of *M. incognita* used for various experiments was propagated from the culture of *M. incognita* maintained in the screen house of the Department of Nematology, CCS HAU, Hisar, on cotton crop. Pure cultures were raised in a screen house in earthen pots filled with sandy loam soil. Before using, the soil was steam sterilized at 15 lb pressure/sq. inch for one and a half hours in an autoclave. After sterilization, the soil was exposed to open air for at least 24h before using for experiments. Seeds of the cotton (H-1098i) were sown in sterilized sandy loam soil in the pots (5 kg soil capacity). J2s of *M. incognita* were obtained from eggs from the pure culture maintained in the department; the seedlings of cotton in pots were inoculated with these J2. The cultures were allowed to multiply for 2-3 generations and were further sub-cultured periodically. The root-knot nematode species was first identified before the propagation of a pure culture.

Five strains of rhizobacteria, namely *B. aryabhattii*, *B. cereus*, *B. atitudinis*, *B. megaterium*, *G. diazotrophicus* were procured from the Department of Microbiology, College of Basic Sciences and Humanities, CCS HAU,

Hisar. For preparing culture filtrates, the bacterial strains were centrifuged at 10,000 rpm for 10 min and the CF was later used in the experiment. The supernatant was passed through a 0.2 mm filter to remove the bacterial cells and the filtrates were collected (1×10^8 cfu/ml).

Eggs of *M. incognita*, were obtained from infected cotton roots by blending and the sodium hypochlorite method. The eggs were placed into each six wells of tissue culture plates (100 eggs) and the measured quantity of stock solution was added to make the resultant dilutions of S/2, S/4 and S/6. S/2 solution was prepared by adding 5 mL of CF and 5 mL of egg suspension. Similarly, S/4 and S/6 were prepared by adding 2.5 and 1.25 mL of CF in 7.5- and 8.75-mL egg suspension, respectively. Sterilized water was taken as a check. Each dilution was replicated three times. These six-well tissue culture plates were kept in a BOD incubator at $25 \pm 0^\circ\text{C}$. The number of juveniles hatched after 1, 2, 4, 6 and 8 days of exposure to different dilutions of different extracts was counted under a stereoscopic binocular microscope. Mean of larvae hatched was calculated.

RESULTS AND DISCUSSION

The effect of culture filtrate (CFs) on egg hatching inhibition of *M. incognita* at three different concentration levels of S/2, S/4 and S/6. The inhibitory effect varied according to rhizobacterial strains, different concentration levels and exposure time. Among five rhizobacterial strains, the maximum hatching inhibition was observed in the case of *B. aryabhattii* (KMT 4), which was 19.9%, followed by *G. diazotrophicus* with 22.1% hatching at S/2 concentration (Table 1). All other rhizobacterial strains significantly reduced egg hatching at all the concentrations as compared to the untreated check (67.5%). The minimum hatching was recorded at a higher concentration of S/2 and it gradually increased with an increase in the dilutions, irrespective of the exposure period. As the exposure period of the tested rhizobacterial strains was increased, the number of emerged juveniles also increased simultaneously. The interaction among the rhizobacterial strains and exposure period was found to be significant at all three concentration levels (S/2, S/4 and S/6). The distilled water taken as control had a significantly higher number of eggs hatched as compared to other treatments. The CFs of rhizobacterial strains showed an inhibitory effect on egg hatching. After 24h of incubation, very little egg hatching was recorded at all the concentrations. Reducing the concentration of CFs showed a positive effect on egg hatching in case of all the rhizobacterial strains. Among the rhizobacterial strains, *B. aryabhattii* showed the highest inhibitory effect, followed by *G. diazotrophicus* at the concentration of S/2 at all the exposure periods. The maximum egg hatching (40.3%) was recorded at S/6 (Table 3), whereas the minimum hatching was seen at S/2 (19.9%). A similar trend was observed with other concentration levels also. The rate of hatching was inversely proportional to the concentration of different rhizobacterial strains at all exposure periods, as it decreased with an increase in all concentrations, viz., S/2, S/4 and S/6.

Table 1. Effect of different rhizobacterial strains on egg hatching of *M. incognita* (S/2 concentration).

Number of juveniles hatched (%) at S/2 concentration						
Rhizobacterial strains (A)	Exposure periods					Mean (B)
	1 st day	2 nd day	4 th day	6 th day	8 th day	
<i>Bacillus aryabhatii</i> KMT4	11.0 (18.9)	16.6 (24.0)	20.6 (26.9)	24.0 (29.2)	27.3 (31.4)	19.9 (26.1)
<i>Bacillus cereus</i> KMT5	25.0 (29.6)	31.6 (34.2)	35.6 (36.6)	40.6 (39.5)	41.3 (39.9)	34.8 (35.9)
<i>Bacillus atitudinis</i> KMS6	17.3 (24.2)	25.6 (30.3)	29.6 (32.9)	33.3 (35.1)	38.0 (37.9)	28.7 (32.1)
<i>Bacillus megaterium</i> KMT8	19.0 (25.6)	23.6 (29.0)	27.0 (31.2)	34.3 (35.8)	35.0 (36.2)	27.7 (31.5)
<i>Gluconacetobacter diazotrophicus</i> 35-47	14.0 (21.6)	19.0 (25.7)	22.6 (28.3)	26.0 (30.5)	29.0 (32.4)	22.1 (27.7)
Untreated check	44.3 (41.7)	54.6 (47.7)	65.6 (54.1)	82.0 (65.3)	91.3 (73.2)	67.5 (56.4)
Mean (A)	21.7 (26.9)	28.5 (31.8)	33.5 (35.0)	40.0 (39.2)	43.6 (41.8)	
C.D. ($p \leq 0.05$)						
Exposure period (B)			(2.9)			
Rhizobacterial strains (A)			(2.6)			
Interaction			(6.5)			
Exposure period v/s Rhizobacterial strains						

Table 2. Effect of different rhizobacterial strains on egg hatching of *M. incognita* (S/4 concentration).

Number of juveniles hatched (%) at S/4 concentration						
Rhizobacterial strains (A)	Exposure periods					Mean (B)
	1 st day	2 nd day	4 th day	6 th day	8 th day	
<i>Bacillus aryabhatii</i> KMT4	15.3 (22.7)	19.0 (25.8)	22.6 (28.3)	26.0 (30.6)	31.0 (33.7)	22.7 (28.2)
<i>Bacillus cereus</i> KMT5	28.6 (32.0)	33.3 (35.2)	36.3 (37.0)	40.3 (39.3)	44.0 (41.5)	36.5 (37.0)
<i>Bacillus atitudinis</i> KMS6	21.6 (27.3)	29.0 (32.5)	35.0 (35.0)	36.3 (37.0)	40.3 (39.3)	32.4 (34.2)
<i>Bacillus megaterium</i> KMT8	23.0 (28.4)	26.3 (30.8)	30.0 (33.1)	32.0 (34.3)	37.3 (37.6)	29.7 (32.9)
<i>Gluconacetobacter diazotrophicus</i> 35-47	19.0 (25.6)	22.3 (28.0)	25.6 (30.4)	29.0 (32.5)	32.6 (34.8)	25.7 (30.3)
Untreated check	44.3 (41.7)	54.6 (47.6)	65.6 (54.1)	82.0 (65.3)	91.3 (73.2)	67.5 (56.4)
Mean (A)	25.3 (29.6)	36.9 (33.3)	35.8 (36.3)	40.9 (39.8)	46.0 (43.4)	
C.D. ($p \leq 0.05$)						
Exposure period (B)			(2.6)			
Rhizobacterial strains (A)			(2.3)			
Interaction			(5.8)			
Exposure period v/s Rhizobacterial strains						

Figures in parentheses are angular transformed values

Table 3. Effect of different rhizobacterial strains on egg hatching of *M. incognita* (S/6 concentration).

Number of juveniles hatched (%) at S/6 concentration Rhizobacterial strains (A)	Exposure periods					Mean (B)
	1 st day	2 nd day	4 th day	6 th day	8 th day	
<i>Bacillus aryabhattii</i> KMT4	20.3 (26.6)	25.3 (30.1)	28.6 (32.3)	32.3 (34.6)	36.3 (37.0)	28.5 (32.1)
<i>Bacillus cereus</i> KMT5	34.0 (35.5)	35.3 (36.4)	40.0 (39.1)	44.6 (41.9)	48.0 (43.8)	40.3 (39.3)
<i>Bacillus atitudinis</i> KMS6	27.3 (31.4)	33.0 (35.0)	37.6 (37.8)	41.6 (40.1)	45.6 (42.4)	37.0 (37.3)
<i>Bacillus megaterium</i> KMT8	28.0 (31.9)	31.0 (33.7)	35.3 (36.4)	39.0 (38.5)	41.6 (40.1)	34.9 (36.1)
<i>Gluconacetobacter diazotrophicus</i> 35-47	25.3 (30.1)	29.6 (32.9)	32.6 (34.8)	36.0 (36.8)	39.6 (39.0)	32.6 (34.7)
Untreated check	44.3 (41.7)	54.6 (47.6)	65.6 (54.1)	82.0 (65.3)	91.3 (73.2)	67.5 (56.4)
Mean (A)	29.8 (32.9)	34.8 (36.0)	39.9 (39.1)	45.9 (42.9)	50.4 (45.9)	
C.D. ($p \leq 0.05$)						
Exposure period (B)			(2.3)			
Rhizobacterial strains (A)			(2.1)			
Interaction			(5.1)			
Exposure period v/s Rhizobacterial strains						

Figures in parentheses are angular transformed values

Nematicidal activity of rhizobacterial strains viz., *B. aryabhattii*, *B. cereus*, *B. atitudinis*, *B. megaterium*, *G. diazotrophicus* was evaluated *in vitro* against egg hatching of *M. incognita* at various dilutions viz., S/2, S/4 and S/6. Among the five rhizobacterial strains, the maximum hatching inhibition was observed in the case of *B. aryabhattii*, which was 19.9%, followed by *G. diazotrophicus* with 2.1% hatching at S/2 concentration. All other rhizobacterial strains significantly reduced egg hatching at all the concentrations as compared to the untreated check (67.5%). After 24h of incubation, very little egg hatching was recorded at all the concentrations. Lowering the concentration of CFs had a positive effect on egg hatching in all the rhizobacterial strains. Among the rhizobacterial strains, *B. aryabhattii* showed the highest inhibitory effect, followed by *G. diazotrophicus* at the concentration of S/2 at all the exposure periods. The maximum egg hatching (40.3%) was recorded at S/6, whereas the minimum hatching was seen at S/2 (19.9%). A similar trend was also observed at other concentration levels. The minimum hatching was seen at a higher concentration of S/2 and it gradually increased with an increase in the dilutions, irrespective of the exposure period. As the exposure period of the tested rhizobacterial strains was increased, the number of emerged juveniles also increased simultaneously. The interaction among the rhizobacterial strains and exposure period was found to be significant at all three concentration levels (S/2, S/4 and S/6). Distilled water taken as control had significantly higher numbers of eggs hatched as compared to other treatments.

These results are in accordance with those reported by Chen *et al.* (2022), where he studied *B. aryabhattii* MCCC

1K02966, a deep-sea bacterium and found certain nematicidal VOCs. Among these VOCs, methyl thioacetate exhibited multiple nematicidal activities, including contact nematicidal, fumigant and repellent activities against *M. incognita*. Also, methyl thioacetate exhibited 80-100% egg-hatching inhibition on the seventh day ranging from 0.5 mg/mL to 5 mg/mL. Abd El-Aal *et al.* (2021) through an *in vitro* experiment on five egg-masses, 100 free eggs and 100 second stage juveniles (J2s) of *M. incognita* showed the nematicidal potential of six strains belonging to *Pseudomonas* spp. and *Serratia* spp. Results showed that the inhibitory effect on hatching of the eggs and juvenile mortality varied according to bacterial species, strains and exposure time. The interaction studies of biocontrol agents and pathogens showed that undiluted culture filtrates of *T. harzianum*, *B. subtilis* and *P. fluorescens* caused 100% mortality against *M. incognita* and inhibited egg hatching up to 75%. *M. incognita* eggs were also found to be infected (up to 89%) when exposed to fresh culture of *T. harzianum*.

The bacterial culture filtrates (CFs) of two *Bacillus* strains had a strong toxic effect on egg hatching and survival of J2s of *M. incognita*. The increased concentration of CFs and incubation period, significantly inhibited egg hatching and also increased the J2 mortality. The crude enzymes which were extracted from culture supernatants of two *Bacillus* strains caused structural damage to the nematode eggs and J2 at 1, 3 and 5 days after inoculation (DAI) (Singh *et al.*, 2021). Soliman *et al.* (2019) isolated six bacterial isolates (*P. aeruginosa*, *Paenibacillus polymyxa*, *Lysinibacillus sphaericus*, *B. cereus*, *B. subtilis* and *Achromobacter xylosoxidans*)

exhibiting high efficacy against root-knot nematodes. These strains produced higher amount of chitosanase, chitinase and protease using colloidal chitin and soluble chitosan as carbon sources. In *in vitro* tests, all the bacterial CFs potentially displayed nematocidal effect on *M. incognita* egg hatching and increased J2 mortality as compared to control. Prevention of egg hatching by rhizobacteria is one of the most studied mechanisms against the PPNs. Reduction in egg hatching may arise due to the stimulation of plant defense systems by the production of plant chitinase and proteases (Seenivasan *et al.*, 2012). Huang *et al.* (2010) showed that PGPR strain *B. megaterium* YMF 3.25 significantly inhibited egg hatching of the nematode and also reduced the infection caused by *M. incognita* through the production of certain nematocidal volatiles. They also confirmed that the nematocidal volatiles produced by the bacterium were mainly benzene acetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl disulphide, which were active against J2s and eggs at the concentration of 0.5 mmol.

CONCLUSION

Based on the findings, it can be inferred that all the bacterial bio-agents that were tested demonstrated the ability to manage root-knot nematode by reducing egg hatching in laboratory conditions. The research overwhelmingly confirms that these bacteria are powerful and versatile biological control agents. Their effectiveness is rooted in a sophisticated, multi-pronged approach that extends beyond simple antagonism. The direct mechanisms of action-which include the enzymatic degradation of the nematode's protective eggshell, the lethal action of crystalline toxins, and the ovicidal effects of potent secondary metabolites-are highly effective at disrupting the nematode life cycle at its most vulnerable stages. Furthermore, *Bacillus* strains confer indirect protection by fostering a healthy root environment and inducing systemic resistance in the host plant, a response that enhances the plant's own natural defenses. The discovery of a synergistic relationship between different nematocidal compounds is a pivotal finding, suggesting that the future of bionematicides lies in developing multi-component formulations that exploit these cooperative effects.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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