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Effect of some Bacterial Bioagents against Root-Knot Nematode (*Meloidogyne incognita* race2)

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Abstract-*The effect of culture filtrate of different bacterial isolates on egg hatching and juvenile mortality of root-knot nematodes* (*Meloidogyne incognita race 2*) *was studied in vitro and the effective strains were selected. The per cent mortality and hatching inhibition was proportional to the concentration of culture filtrate and the duration of exposure period. Culture filtrate of all the isolates of bacteria significantly induced mortality and inhibition of egg hatching of M. incognita juveniles. The highest percentage of inhibition of egg hatching was recorded for Bacillus thuringiensis followed by Bacillus sp. and Pseudomonas fluorescens whereas the highest percentage of mortality of juvenile was recorded for Bacillus thuringiensis followed by Pseudomonas fluorescens and Pseudomonas sp.*

Keywords: Rhizospheric bacteria, egg hatching, juvenile mortality, culture filtrate, duration of exposure period, root-knot nematode.

I. INTRODUCTION

Root-knot nematode *Meloidogyne incognita* race 2 is one of the major constraints in crop production in Assam. Present strategies for nematode management largely depend on cultural practices such as crop rotations, use of resistant varieties and use of nematicides which has several disadvantages. Utilization of biocontrol agent is of advantage over the above mentioned nematode management strategies. Many species of *Pseudomonas* and *Bacillus* have been reported as plant growth promoting rhizobacteria (PGPR) which produces iron-chelating siderophores, antibiotics or hydrogen cyanide, and these compounds have been implicated in the reduction of deleterious and pathogenic rhizosphere microorganisms, creating an environment more favourable for root growth[1] .It has been demonstrated that bio-agents produce different metabolites and antibiotics which directly or indirectly stimulate plant growth [2] . A number of bacterial species has been used as biocontrol agents against *Meloidogyne* spp. [3-12]. However, few biocontrol products are currently commercially available and it is necessary to find different strains of antagonistic bacteria for controlling plant-parasitic nematodes more efficiently. Therefore, a laboratory bioassay was carried out to test some of the isolated strains of *Bacillus* and *Pseudomonas* for their nematicidal activity.

II. MATERIAL AND METHODS

Isolation of bacteria

A survey was conducted during Feb 2018 to May 2018 in different localities of the district Jorhat, Assam comprising an area approximately 500 ha, in order to identify the nematodes infection and to isolate the bacterial antagonists from crops rhizosphere viz. cucurbits, tomato, brinjal, okra, cabbage, banana, citrus, and tea. A total of 50 soil (500 g) sample were collected from the rhizosphere of each crop. Serial dilution technique was used for the isolation of bacterial strain. One g of rhizosphere soil was dispensed in 9 ml sterile water, from the 10^{-6} dilution, 50 µl were inoculated over Petri plates containing Nutrient Agar and King's B agar media (autoclaved at 121°C for 15 min) separately. The plates were incubated at room temperature 28±2°C for 48-72h.The colonies obtained on plates were picked and streaked on more plates and purified by re-streaking. The isolates were initially categorized into two broad groups based on Gram staining by Hucker's modified method [13]. The isolated strains were identified by up to generic level by Bergey's manual and using standard protocol [14, 15].Two isolates namely *Pseudomonas* sp. and *Bacillus* sp. were collected from soil rhizosphere of cowpea and banana respectively. *Pseudomonas fluorescens, Bacillus thuringiensis, B.brevis* were procured from the Department of Plant Pathology, AAU, Jorhat, Assam. All the strains were evaluated for nematicidal properties against nematodes.

Nematode inoculum and mass culturing

The inoculum of root-knot nematode *M. incognita* race 2 was collected from naturally infested tomato crop in field and single egg mass was used to raise pure culture. Mass culturing of nematodes was done on tomato variety Sel 7, in

order to get regular supply of the inoculums for the experiments. One month old tomato seedlings were inoculated with small volume of egg suspension approximately consisting of 2000 eggs of *M.incognita* race 2. These pots were watered and kept in glasshouse at temperature 28-35°C.

Preparation of culture filtrate and test for nematoxicity of bacterial culture filtrate

The bacterial isolates were inoculated in the respective medium (40 ml). *Pseudomonas* on King's B broth and *Bacillus* on Nutrient broth were separately incubated at 30°C on a shaker for 48 hrs. The liquid culture was filtered through Whatman No.1 filter paper and passed through bacterial filter[16] .Filtrates were centrifuged at 6000 rpm for 15 min. The supernatant was taken and the suspended residue was discarded.

Juvenile mortality test

One ml of S/2, S/8, S/32 dilutions of each cell-free culture filtrates was mixed with 1 ml nematode suspension (containing ca. 100 *M.incognita* J2) in cavity block to obtain S/4, S/16 and S/64 dilutions and tested for per cent mortality of nematodes. There were 3 replications for each treatment. Observations were taken after 24 h, 48 h and 72 h. The immobile J2 were confirmed for mortality by randomly disturbing with a needle. Sterile water and medium alone served as control. The per cent juvenile mortality was calculated by the following formula.

$$\text{Percent mortality} = \frac{\text{Total number of dead juveniles}}{\text{Total number of juveniles}} X\ 100$$

Egg hatching test

To determine the effect of culture filtrate on the hatching of eggs of J_2 of *M. incognita* eggmasses of *M.incognita* were dissolved in NaOCl to release individual eggs [17]. Sterilized Petri dishes of 5 cm dia were separately pipette 1 ml of culture filtrate with dilution S/2, S/8 and S/32. Hundred eggs of *M.incognita* in 1 ml water suspension were transferred to each dish. Medium alone and water alone served as control. All Petri dishes were kept at 28±2°C, replicated thrice. Observations were recorded on after 5, 10 and 15 days. The per cent egg hatch was calculated by the following formula.

$$\text{Hatching \%} = \frac{\text{No. of hatched juveniles}}{\text{No. of hatched} + \text{unhatched eggs}} X\ 100$$

Statistical analysis

Per cent egg hatch and per cent mortality data was subjected to statistical analysis using the three factorial completely randomized design statistical package. The critical differences in main effects i.e. isolates, concentration, and time of exposure as well as in their interactions were tested at P=0.05.

III. RESULTS

Two numbers of isolate of bacteria were isolated from the root rhizosphere of *M.incognita* race 2 infected plants. *Bacillus sp.* and *Pseudomonas* sp. were isolated from rhizospheric soil of banana and brinjal, respectively. The results presented revealed significant differences in juvenile mortality of *M.incognita* among isolates (biocontrol agent) (T), concentration of culture filtrate(C) and exposure period (t).

The data showed in the Table 1 revealed that all the culture filtrates of bacterial isolates were having nematicidal effect of varying degree on *M.incognita* race 2. Per cent mortality of nematodes was directly proportional to the concentration of culture filtrate and the period of exposure. Irrespective of concentration of culture filtrate(C) and duration of exposure (t), six isolates namely *Bacillus* sp., *B.brevis B.thuringiensis*, *Pseudomonas* sp., *P.florescence* and *P.aeruginosa* were exhibited nematicidal effects on *M. incognita* J_2. Irrespective of concentration of culture filtrate and exposure period the mortality rates of *M. incognita* J_2 caused by *B.thuringiensis* reached 75.77% at which is at par with *P.florescence* (75.44%).All the new isolate namely *Bacillus* sp, and *Pseudomonas* sp displayed more than 40% juvenile mortality during 24 h exposure time at S/16 concentration of culture filtrate. On the other hand, *P.aeruginosa* showed the lowest toxicity, caused only 48% juvenile mortality S/64 at concentration of culture filtrate during 48 h exposure time. Irrespective of isolate and period of exposure the mortality rate of *M. incognita* J_2, at lowest concentration (S/64) is 43.62% whereas at highest concentration (S/4) mortality rate is 51.66%.Similarly irrespective of isolate and culture filtrate concentration the mortality rate of *M. incognita* J_2 is highest (63.07%) at 72 h exposure period and lowest (34.85%) at 24h exposure period. *B.thuringiensis* and *P.florescence* showed highest toxicity (100% J2 mortality) at S/16 concentration of culture filtrate during 72 h exposure period. There was least effect of media on juvenile mortality of *M. incognita*.

The results presented in Table 2, revealed significant differences among isolates (T), concentration of culture filtrate(C) and exposure period (t) in egg hatching of *M. incognita*. Irrespective of concentration of culture filtrate (C) and time of exposure period (t), the culture filtrate of *B.thuringiensis* followed by, *Bacillus* sp., *P. fluorescens* and *Pseudomonas* sp. adversely affected the larval hatching of

M.incognita race 2. Similarly, irrespective of isolate (T) and concentration of culture filtrate (C), time of exposure (t) also affected the larval hatching. With increase in exposure period up to 15 days there was a correspondingly increased in egg hatching. Hatching of *M. incognita* J_2 is highest (57.03%) at 15 day exposure period and lowest (40.00%) at 5 day exposure period. With increase in the dilution of culture filtrate, the cumulative hatching was increased irrespective of isolate (T) and time of exposure period (t). Highest inhibition in hatching was obtained in S/4 concentration of each bacterial culture filtrates. The percentage hatching of *M.incognita* was 38% for 15 day of exposure period in the S/64 concentrations of culture filtrates of *B.thuringiensis* followed by *Pseudomonas* sp. with percentage hatching of 48. *B.brevis* and *P.aeruginosa* showed negligible effect on inhibition of egg hatching of *M.incognita*. There was negligible effect of culture media on hatching of *M.incognita*.

IV. DISCUSSION

Culture filtrates of several soil borne microbes are known to exhibit nematicidal action besides inhibiting larval emergence of plant parasitic nematodes. Antibiotics, extracellular enzymes and other toxic compounds present in metabolites of rhizobateria are probably involved in the increase of J_2 mortality and the inhibition of egg hatching[18]. The lethal effect of the cultural filtrates of these bacteria may be attributed to the production of nematicidal metabolites i.e. lytic enzymes (gelatinase, protease and chitinase) and volatile compounds in the cultural media. Similar results were reported by Ali *et al.* [19] who found that cultural filtrates of *Pseudomonas* sp. caused juvenile mortality of *M. javanica*. The nematicidal volatile products that were produced by *Bacillus* and were characterized to include mainly the benzene acetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl disulphide, which were active against *M. incognita* juveniles and eggs at the concentration of 0.5 mmol [20]. Ahl *et al*., [21] proposed the mechanism of reduction of nematode population which was due to premature egg hatching and reduction in viability and mortality of juveniles induced by secondary metabolites such as 2,4 Diacetylphloroglucinol and lytic enzymes [22], antibiotics and hydrogen cyanide produced by *Pseudomonas* spp. and non cellular extract and toxic metabolites like bacillopeptidase, subtillin E and a lactamase from *Bacillus* spp. Regina *et al*. [23] and Hanna *et al*. [24] have reported that mortality of *M. incognita* increased with increase in exposure time as well as the concentration. The effects of rhizobacterial toxins include the suppression of nematode reproduction, egg hatching and juvenile survival, as well as direct killing of nematodes by causing paralysis and convulsive movements [4]. Dhawan *et al.* [25] evaluated four strains of *Bacillus thuringiensis*, and found that the mobility of *M.incognita* juveniles completely ceased after 24h exposure in S and S/10 dilutions. However, all dilutions above S/25 were ineffective. Production of surfactin by *Bacillus* is a characteristic that supports their persistence under extreme conditions [26, 27]. El-Hamshary *et al.* [28] found that *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* affected *M. incognita* juvenile survival *in vitro* study, and the mortality percentages of the nematode were dependent on the bacterial concentration and exposure time. Terefe *et al.* [29] found that an aqueous suspension of *Bacillus firmus* at 2.5 and 3% concentration caused 100% inhibition of mobility *M. incognita*, 24 hrs after treatment. The mortality percentage of *Bacillus circulans* (KSB2) at dilutions of 1/100 and 1/1000 were the highest inhibitors comparing with the other tested bacteria being 97.8 and 40.3%, respectively [30]. Xia *et al.* [31] reported that the culture supernatant of *B. subtilis* strains caused a higher mortality of *M. incognita* J_2. Nagesh *et al.* [32] observed that, cell-free culture filtrates of *B. cereus* reduced egg hatching (90%) and caused 100% mortality of juveniles. Bin *et al.* [33] mentioned that culture filtrates of rhizobacterium are heat stable and resistant to extreme pH values, which suggested that the antibiotic action might be responsible for the nematicidal activity**.** The exposure of *M. incognita* to various concentrations (5-100%) of cultural filtrate of *Paenibacillus polymyxa in vitro* conditions significantly reduced egg hatching and caused substantial mortality of its juveniles [34]. These results are similar with the findings of Ying *et al.* [35] who reported *Bacillus* spp. culture could significantly inhibiting the hatching of eggs and increases the mortality of second stage juveniles and reduce infection of the nematode through production of nematicidal volatiles. Antibiotic production viz., 2, 4 DAPG, phenazine and pyocyanine were also well pronounced in the *Pseudomonas* strain, Pf 128 through thin layer chromatography [36] .The failure to regain its activity of *M. incognita* after separating from culture filtrate of *Pseudomonas* spp. placed over sterile water demonstrates the numbers of metabolites that had longer systemic activity [37,38].

V. CONCLUSION

It is clear from this work that, there are many bacteria which have potentialities for controlling root-knot nematode in plant rhizosphere region. Among six bacterial isolates *Bacillus thuringiensis and Pseudomonas fluorescens* were

exhibited the highest production of nematicidal activities against root-knot nematode *M. incognita* race 2.

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Table.1: Effect of culture filtrate of some bacterial bioagent on juvenile mortality of Meloidogyne incognita race 2.

Treatment	Culture filtrate concentration	Period of exposure(h)			Treatment (T)Mean	Culture filtrate concentration (C)Mean
		24	48	72		
Bacillus sp.	S/64	36(36.85)	55(47.87)	85(67.21)	62.00(52.79)	43.62(39.61)
	S/16	40(39.20)	58(49.62)	86(68.08)		48.85(44.26)
	S/4	48(43.85)	60(50.79)	90(71.62)		51.66(46.20)
B.brevis	S/64	38(38.04)	60(50.79)	70(56.79)	63.44(53.81)	
	S/16	42(40.38)	62(51.94)	90(71.94)		
	S/4	50(44.99)	66(54.33)	93(75.05)		
B.thuringiensis	S/64	55(47.86)	74(59.35)	75(60.00)	75.77(63.74)	
	S/16	60(50.76)	77(61.38)	100(90.00)		
	S/4	62(51.94)	79(62.74)	100(90.00)		
Pseudomonas sp.	S/64	46(42.70)	60(50.76)	72(58.11)	66.44(55.87)	
	S/16	50(44.99)	62(51.94)	93(74.34)		
	S/4	52(46.14)	68(55.64)	95(75.07)		
P.florescence	S/64	58(49.60)	74(59.35)	76(60.67)	75.44(63.52)	
	S/16	58(49.60)	75(60.00)	100(90.00)		
	S/4	60(50.76)	78(62.08)	100(90.00)		
P.aeruginosa	S/64	38(38.04)	48(43.85)	82(64.91)	59.55(51.13)	
	S/16	42(40.39)	52(46.14)	84(66.44)		
	S/4	48(43.85)	56(49.02)	86(68.05)		
Culture media(NA)	S/64	6(14.04)	10(18.37)	20(26.54)	14.00(21.49)	
	S/16	8(16.34)	12(20.7)	20(16.50)		
	S/4	10(18.37)	18(25.07)	22(27.94)		
Culture media(KB)	S/64	8(16.34)	10(18.37)	16(23.54)	14.00(21.75)	
	S/16	12(20.22)	12(20.22)	18(25.07)		
	S/4	14(21.93)	16(23.46)	20(26.54)		
Distilled water	S	0(0.00)	2(8.13)	4(11.27)	1.77(6.12)	
	S	0(0.00)	2(8.13)	4(11.27)		
	S	0(0.00)	2(8.13)	2(8.13)		
Period of Exposure (t)Mean		34.85 (33.60)	46.22 (41.38)	63.07 (55.09)		
CV=4.47,CD(P=0.05): Treatment(T):1.03; Concentration(C):0.59; Period of exposure (t): 0.59;T×C: 1.79; T×t: 1.79; C×t:1.50;T×C×t : 3.10						

Figures in the parentheses are Arc-Sine transformed values

Table.2: Effect of culture filtrate of some bacterial bioagent on hatching of Meloidogyne incognita race 2.

Treatment	Culture filtrate concentration (%)	Period of exposure(days)			Treatment Mean	Culture filtrate concentration Mean
		5	10	15		
Bacillus sp.	S/64	32(34.43)	38(38.04)	52(46.14)	35.33 (36.32)	52.25(46.61)
	S/16	26(30.65)	34(35.66)	44(41.54)		48.70(44.35)
	S/4	22(27.94)	32(34.43)	38(38.04)		45.74(42.47)
B.brevis	S/64	39(38.63)	48(43.85)	56(48.44)	43.11 (41.01)	
	S/16	30(33.19)	45(42.70)	52(46.14)		

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	S/4	28(31.93)	40(39.22)	50(44.99)		
B.thuringiensis	S/64	22(27.95)	30(33.19)	38(38.04)	26.55 (30.77)	
	S/16	18(25.07)	26(30.64)	36(36.86)		
	S/4	15(22.71)	22(27.95)	32(34.43)		
Pseudomonas sp.	S/64	30(33.19)	40(39.22)	48(43.85)	37.77 (37.87)	
	S/16	36(36.86)	38(38.04)	44(41.55)		
	S/4	32(34.43)	32(34.43)	40(39.22)		
P.florescence	S/64	34(35.66)	42(40.39)	50(44.99)	36.66 (37.09)	
	S/16	24(29.32)	38(38.04)	46(42.70)		
	S/4	22(27.95)	30(33.19)	44(41.55)		
P.aeruginosa	S/64	34(35.66)	44(41.54)	50(44.99)	38.66 (38.32)	
	S/16	28(31.93)	38(38.03)	48(43.85)		
	S/4	24(29.31)	36(36.85)	46(42.70)		
Culture media(NA)	S/64	64(53.12)	78(62.03)	82(64.91)	72.33 (58.47)	
	S/16	63(52.53)	74(59.35)	80(63.44)		
	S/4	60(50.76)	72(58.05)	78(62.03)		
Culture media(KB)	S/64	67(54.93)	78(62.03)	82(64.93)	73.55 (59.24)	
	S/16	64(53.12)	76(60.66)	80(63.44)		
	S/4	62(51.94)	75(60.00)	78(62.03)		
Distilled water	S	70(59.02)	79(62.72)	84(66.49)	76.11 (61.19)	
	S	67(54.93)	78(62.16)	82(64.91)		
	S	67(54.95)	78(62.03)	80(63.44)		
Period of Exposure (t)Mean		40.00 (38.97)	49.66 (44.98)	57.03 (49.47)		
CV=3.33,CD(P=0.05): Treatment(T):0.78; Concentration(C):0.45; Period of exposure (t): 0.45;T×C: 1.35; T×t: 1.35; C×t:0.78;T×C×t : 2.34						

Figures in the parentheses are Arc-Sine transformed values