BIOCONTROL OF *MELOIDOGYNE GRAMINICOLA* USING RHIZOBACTERIA ON RICE SEEDLINGS

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Summary. The potential of seven rhizobacterial strains to prevent attacks of rice cv. Basmati-370 seedlings by the rice root-knot nematode, *Meloidogyne graminicola*, was evaluated in screenhouse tests. In *in planta* tests, all the rhizobacteria were found to possess significant activity against *M. graminicola*. Seed inoculation with *Gluconacetobacter diazotrophicus* Co99-70 was the most effective in preventing J_2 penetration, by up to 92%. *Bacillus* sp. RKB-91, *G. diazotrophicus* Co99-70 and *Pseudomonas* sp. RKP-33 were highly effective in reducing galling severity (by more than 80%) and in lowering nematode multiplication. These bacteria also promoted root and shoot growth of seedlings. In an *in vitro* test, exposure to culture supernatant of *G. diazotrophicus* Co99-70, *Pseudomonas* sp. RKP-33, and *Bacillus* sp. RKB-91 significantly delayed and decreased egg hatching and irreversibly inactivated motility of infective juveniles of the nematode by 64%, 89% and 77%, respectively, within 24 h of exposure.

Keywords: *Bacillus* sp., *Gluconacetobacter diazotrophicus*, *Oryza sativa* L., *Pseudomonas* sp., rice root-knot nematode.

The rice root-knot nematode, *Meloidogyne graminicola* Golden *et* Birchfield, is a severe pest of rice (*Oryza sativa* L.) in several rice producing areas and particularly in nursery beds, where flooding is intermittent or absent. Recently, severe infestations of *M. graminicola* were identified in several rice nursery seedbeds in Bangladesh, using a soil bioassay test (Padgham, 2003; Padgham *et al.*, 2004a). Also, Padgham *et al.* (2004b) reported significant increases in seedling height and shoot dry weight of rice grown in seedbeds treated with carbofuran. The use of rice seedlings from non-treated nursery beds has resulted in yield loss of rice grain of 38% in comparison to 29% when rice seedlings from treated nursery beds were used (Gaur, 2003), thus suggesting the importance of controlling nematodes in nursery beds. In addition, transplanting seedlings from infested and non-treated nurseries would spread the nematodes in farmers' field. Rhizobacteria, especially plant growth promoting bacteria, are considered ideal candidates for nematode control because of their antibiosis effects, due to the nematotoxic metabolites produced during their growth (Castro *et al.*, 1990; Bansal and Bajaj, 2003) and also due to their high rate of multiplication, spread and colonization of the rhizosphere and plant root surface (Dobbelaere *et al.*, 2003).

In the light of the above information, an investigation was undertaken to evaluate the biocontrol potential of three strains of *Gluconacetobacter diazotrophicus* Yamada, Hoshino *et* Ishikawa, one of *Azotobacter chroococcum* Beijerinek, one of *Pseudomonas* sp. and two of *Bacillus* spp., *in vitro* and as seed inoculation, against *M. graminicola* infecting rice seedlings in a screenhouse, to simulate nursery bed conditions.

MATERIALS AND METHODS

The seven bacterial strains used in this study (*G. diazotrophicus*, Co99-70, 35-47 and 767-50; *A. chroococcum* AVK-51; *Pseudomonas* sp. RKP-33 and RKB-91 and RKB-65 of *Bacillus* spp) were obtained from the culture collection of the Department of Microbiology, CCS HAU, Hisar. All bacterial cultures were maintained on their respective medium slants, i.e. LGI (Lacto-glucose infusion) for *Gluconacetobacter*, Jensen's for *Azotobacter*, King's B for *Pseudomonas* and LB (Luria Bertini) for *Bacillus*. Bacteria-free culture supernatants (BFCS) of the strains were assayed for their effect on egg hatching and juvenile motility of *M. graminicola*. For this, each strain was grown in its respective medium broth for 4 days at 30 °C in a BOD incubator and then centrifuged at 10,600 g for 20 min. The bacteria-free supernatants thus obtained were stored at 5 °C until used.

Effects of BFCS on egg hatching. For the extraction of eggs, nematode infected rice plants were uprooted from pots. Roots were washed with tap water and galls were separated with a dissection blade and macerated in a ceramic mortar and pestle in distilled sterile water (DSW). This suspension was sequentially passed through 150, 53, and 30 µm pore size sieves. The residue retained on the 30 µm sieve was collected after washing five times and used immediately. Samples (2 ml) of each BFCS were placed in solid watch glasses and, to each of these, 0.2 ml of suspension, containing about 250 nematode eggs was added. Watch glasses were covered with glass slides to reduce evaporation and incubated at 30 °C. Emerging juveniles were counted after 0, 2, 4, 6, 8 and 10 days of exposure. Per cent hatches were calculated with reference to the average number of eggs of the

replicates of each treatment. Distilled water and the various media without BFCS were used as controls. After 10th day of incubation the eggs were placed on a 30 µm sieve and washed five times with DSW to remove BFCS and then further incubated in distilled water for another three days to observe the resumption of hatching, if any. Each treatment was replicated three times.

Effect of BFCS on juvenile motility. A suspension of juveniles obtained after two days of incubation of the eggs was prepared in DSW and used immediately. About 150 juveniles (0.2 ml of suspension) and 2 ml of BFCS were transferred to each well of tissue culture plates. Sterilized distilled water and the various media were included as controls and each treatment was replicated three times. Plates were kept at 30 °C. Each well was examined microscopically at 0, 24, 48, and 72 h and juveniles that appeared straight and motionless, and that did not respond to a touch with a needle, were considered non-motile and counted. Non-motile juveniles were washed free of BFCS and transferred to DSW and observed after two more days to ascertain whether or not they resumed motility.

Effects of rhizobacteria on plant growth and root infection by juveniles. To study the effect of rhizobacterial inoculation on juvenile penetration of rice seedlings grown in pots, surface-sterilized (by immersing in 2% sodium hypochlorite solution for 5 min. and subsequently rinsing twice in DSW) rice cv. Basmati-370 seeds were immersed in bacterial culture suspension (3.0×10^8 cells/ml) of each strain separately for 30 minutes and sown in earthen pots (50 seeds/pot; 15 cm diameter pots) filled with 1 kg sandy loam textured soil naturally infested with *M. graminicola* at a density (Pi) of 108 J_2/100 g soil. Each treatment was replicated three times and the pots were arranged in a randomised block design on benches in a screenhouse. Quarter strength sterilized Sloger's nitrogen free salt solution was used for irrigating the pots. The *pro rata* recommended dose of fertilizers (N = 150 kg/ha and P = 60 kg/ha) was applied. Two seedlings from each pot were chosen at random and uprooted gently at 2, 4, 6, 8 and 10 days after seed germination (when seedlings appeared above the soil surface). Then the roots were washed gently, stained in boiling lactophenol solution containing 0.05% acid fuchsin for one minute, and destained by immersing in clear lactophenol solution until maximum contrast was developed. For counting the J_2 that had penetrated the roots, the whole root system was spread on a glass slide and teased open by pressing with a needle. Juveniles present inside the root were counted and recorded as the number of juveniles penetrated/root system. To further observe the effect of rhizobacterial inoculation on root-knot disease intensity and plant growth components, 45 days after sowing, shoot and root weights (average of ten plants from each pot) per plant, number of galls/root system, and nematodes (all stages including eggs) per root system were all measured. Final soil nematode (J_2) densities were recorded after using a decantation and sieving method for their extraction. For this, the soil of each pot was mixed thoroughly and a 100 g sample from each pot was vigorously mixed with tap water in a pan before passing the suspension through 250 and 150 µm sieves to remove soil and plant debris. Each soil sample was washed and decanted three times. Subsequently, the suspension was passed through a 30 µm sieve. The residue retained on the 30 µm sieve was suspended in 30 ml DSW and placed on tissue paper supported on aluminium mesh in a Petri plate (10 cm diameter). After 3 days of incubation at 30 °C, the juveniles that passed through the tissue paper were counted under a binocular microscope.

Statistical analysis. Per cent values of juvenile hatch and non-motile juveniles were subjected to angular transformation before analysis of variance. The data from the screenhouse experiment were analyzed as a completely randomized design (CRD) and critical differences at P = 0.05 were calculated to compare the treatments.

RESULTS

Effects of BFCS on egg hatching. In general, exposure of *M. graminicola* eggs to the bacteria-free culture supernatant of various strains of rhizobacteria significantly suppressed egg hatch (Table I). The cumulative percent hatch ranged between 20.1 and 36.7 over a period of ten days of incubation compared to 96.4% in water. Furthermore, hatching decreased with time of incubation and completely ceased after 2 days of incubation in *Bacillus* sp. RKB-91 and RKB-65 and after 4-6 days in the other samples of BFCS. When unhatched eggs were transferred to water, hatching did not resume if they had been previously incubated in BFCS of *Bacillus* sp. RKB-91, while it resumed up to 63.3% if previously incubated in BFCS of *G. diazotrophicus* 35-47, suggesting that the various BFCS behaved differently and may have affected nematode embryonic development.

Effect of BFCS on juvenile motility. Of the juveniles exposed to culture supernatants a significantly large proportion (30.9%-89.0%) became non-motile within 24 h of incubation, whereas all the juveniles exposed to water and most of those exposed to the various growth media remained active (data not presented here) during this period. None of the juveniles immobilised in the various BFCS resumed motility when transferred to water.

Effects of rhizobacteria on plant growth and root infection by juveniles. The screenhouse experiment indicated that all the rhizobacterial strains reduced significantly the penetration by juveniles of the roots of rice

Table I. Effects of bacteria-free culture supernatants of various rhizobacteria on egg hatching and juvenile motility of *Meloidogyne graminicola*.

Strain	Effect on J_2		Effect on eggs	
	% non motile within 24 h in BFCS	% resumed motility in water	% hatched during 10 days in BFCS	% hatched during 3 more days in water
Water	0.0 -	-	96.4 (75.6)	-
G. diazotrophicus				
Co99-70	64.3 (53.4)	0.0	24.1 (21.1)	34.6
35-47	46.2 (42.7)	0.0	36.7 (31.2)	63.3
767-50	30.9 (33.8)	0.0	28.7 (24.6)	25.0
A. chroococcum				
AVK-51	44.4 (41.5)	0.0	28.6 (24.6)	25.0
Pseudomonas sp.				
RKP-33	89.0 (72.6)	0.0	20.1 (15.5)	16.2
Bacillus sp.				
RKB-91	77.4 (61.8)	8.3	29.3 (26.1)	0.0
RKB-65	71.4 (57.6)	0.0	34.5 (29.8)	13.9
CD at 5%	- (6.4)	-	- (3.1)	

Figures in brackets are corresponding angular transformed values.

Table II. Effect of rhizobacteria inoculation on penetration of *M. graminicola* juveniles in rice seedlings.

Treatment	Number of J_2 penetrated/root system at different time intervals after seed germination (days)					% average reduction over untreated check
	2	4	6	8	10	
Untreated check	29.8	23.0	30.8	32.4	32.2	-
G. diazotrophicus						
Co99-70	2.0	1.6	2.4	2.5	2.4	92.6
35-47	11.2	12.6	11.2	11.6	14.6	54.7
767-50	3.6	4.4	4.7	4.9	3.8	88.2
A. chroococcum						
AVK-51	10.0	12.2	12.4	14.8	12.8	60.2
Pseudomonas sp.						
RKP-33	3.4	3.7	3.8	4.2	3.9	87.9
Bacillus sp.						
RKB-91	4.2	6.2	6.8	6.8	6.6	79.5
RKB-65	3.4	3.8	4.4	4.4	3.8	88.2
CD at 5%	2.1	1.9	2.8	2.0	4.9	-

seedlings. The percent reduction in infection ranged from 54.7% to 92.6% (Table II). The strain *G. diazotrophicus* Co99-70 was the most effective as it prevented nematode root infection by up to 92.6%, with only 2.4 J_2/root system in comparison to 32.2 J_2 in the untreated check on day 10 after seed germination. The number of juveniles per root system at different time intervals revealed that fewer J_2 could penetrate during the early growth of seedlings treated with rhizobacteria in comparison to the untreated check. On the second day, the average number of J_2 per root system was 2.0, 3.6, 3.4 and 4.2 in *G. diazotrophicus* Co99-70 and 767-50, *Pseudomonas* sp. RKP-33 and *Bacillus* sp. RKB-91, respectively, against 29.8 J_2 per root system in the untreated check. All of the seven strains used in the screenhouse trial significantly improved root and shoot growth of the rice seedlings in the infested soil (Table III), with strain Co99-70 performing best.

In addition to plant growth improvement, bacterial inoculation also decreased root galling by 42.4% to 86.4% relative to the untreated check. Nematode population densities per root system and per pot at harvest decreased significantly in all the treatments compared to the untreated check. On average, with inoculation of *G. diazotrophicus* Co99-70 and 767-50, *Pseudomonas* sp. RKP-33 and *Bacillus* sp. RKB-65, the initial nematode population (Pi) multiplied by 5.6, 5.9, 5.7 and 4.9 times, respectively, compared to a reproduction rate of 40.9-fold in the untreated check (Table III).

Table III. Effect of rhizobacteria as seed inoculation on *M. graminicola* multiplication and seedling growth of rice cv. Basmati-370.

Treatment	Nematode galls/plant	Final nematode population			Reproduction factor (Pi:Pf)	Plant shoot weight (g)	Plant root weight (g)
		In 100 g soil (A)	Per root (B)	Total (Pf) (A+B)			
Untreated check	24.3 -	1386	3011	4397	1:40.9	0.24	0.13
G. diazotrophicus							
Co99-70	4.3 (82.3)	487	117	604	1:5.6	0.70	0.59
35-47	14.0 (42.4)	1444	752	2196	1:20.4	0.51	0.47
767-50	5.7 (76.5)	408	225	633	1:5.9	0.35	0.26
A. chroococcum							
AVK-51	6.3 (72.8)	650	179	829	1:7.7	0.38	0.28
Pseudomonas sp.							
RKP-33	4.7 (80.6)	333	284	617	1:5.7	0.37	0.27
Bacillus sp.							
RKB-91	3.3 (86.4)	476	166	642	1:5.9	0.34	0.23
RKB-65	3.3 (86.4)	410	188	528	1:4.9	0.37	0.36
CD at 5%	7.7 -	96	110	-	-	0.10	0.12

Data in brackets denote percent decrease over untreated check.

DISCUSSION

Increased nematotoxic effect of culture supernatants of various rhizobacteria in comparison to the growth media (LGI, LB, Jensen's and King's B) used in the present study indicated the presence of antinemic metabolites produced by bacteria during their growth. This is understandable because several metabolites of microbial origin have been reported to possess antibiosis effects against nematodes. Volatile fatty acids produced by *G. diazotrophicus* (Bansal *et al*., 2005) are known to disrupt (Djian *et al*., 1991) and paralyse the movement of nematode. Moreover, these organic acids also reduce egg hatching by impairing embryogenesis of *M. incognita* (Bansal and Bajaj, 2003). Delayed nematode egg hatch and reduced motility of J_2 of *Meloidogyne* spp. due to culture supernatants of *Pseudomonas* sp. (Sharma *et al*., 1998) and *Bacillus* sp. (Padgham *et al*., 2005) are also known, and rice seed inoculation with rhizobacteria significantly reduced the penetration of the juveniles of *M. graminicola*, galling severity and nematode multiplication. Similar alleviation of nematode disease intensity has been exhibited by a number of rhizobacteria. *Pseudomonas fluorescens* Fluge and *B. megaterium* de Bary were found to reduce root galling and J_2 penetration by *M. graminicola* in rice seedlings (Anita and Rajendran, 2005; Padgham *et al*., 2005). Reduced nematode penetration and root galling in rhizobacteria-inoculated seedling is probably related to the nematoxic metabolites produced by these bacteria, which reduced egg hatch and motility of juveniles. Thus, fewer J_2 were available to invade host plant roots, producing fewer root galls. Further, disruption in the movement of infective juveniles due to fatty acids (Djian *et al*., 1991), reduced attractiveness by root tips due to ammonia (Castro *et al*., 1990) excreted by these bacteria, and the ability of rhizobacteria to colonize root surface (Dobbelaere *et al*., 2003) probably delayed and reduced root invasion and egg deposition, resulting in reduced numbers of nematodes per root system and thus affecting the total reproduction in comparison to the untreated check.

The beneficial effects on seedling growth given by the rhizobacteria would mainly be due to two reasons. Firstly, plant growth was enhanced due to reduction in root knot disease intensity. Secondly, the nutritional status of the growing seedlings was directly enhanced by providing nitrogen (Dobbelaere *et al*., 2003). Also, rhizobacteria help in acquiring phosphorus and potassium from soil, mainly through their effects on root morphology and physiology (Cocking, 2003).

Additionally, improvement in plant growth can also be attributed to the root hormone that makes the plant grow faster with a better developed root system than non-bacterised plants. Gibberellic acid, which is primarily responsible for stem elongation, is produced by *Azotobacter* spp. (Martinez-Toledo *et al*., 1988), *G. diazotrophicus* (Bastian *et al*., 1998) and *Bacillus* spp. (Gutierrez-Manero *et al*., 2001).

Because of all the above, it can be concluded that *G. diazotrophicus* Co99-70 and 767-50, *Pseudomonas* sp. RKP-33, and *Bacillus* sp. RKB-91 have high activity against *M. graminicola* in controlled laboratory and screenhouse tests. Therefore, inoculation of seeds with these bacteria may significantly reduce J_2 penetration and root galling and thus protect root systems against *M. graminicola* during the early growth stage of rice seedlings. Padgham *et al*. (2004b) demonstrated that *M. graminicola* is capable of substantial growth reduction of rice seedlings in nursery seedbeds and that rice yield increased by 1.0 t/ha where carbofuran was applied to the seedbed and main field, emphasizing the necessity of nematicide application to the field plots also when

transplanting from the carbofuran-treated nursery. This is perhaps obvious because, being a biodegradable chemical, the efficacy of carbofuran on *M. graminicola* declines 20 days after its application in paddy (Krishna-Prashad and Rao, 1982). Contrary to this, the rhizobacteria remain alive and continue to enhance growth in response to developing plant roots (Kloepper and Beauchamp, 1992). The effective cultures from this work will be tested in *M. graminicola* infested nursery beds and main fields during the ensuing rice crop season for the confirmation of results.

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