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Suppression of *Meloidogyne javanica* by antagonistic and plant growth-promoting rhizobacteria*

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Abstract: Four rhizobacteria selected out of over 500 isolates from rhizosphere of the vegetables in China were further studied for suppression of the root-knot nematode and soil-borne fungal pathogens in laboratory and greenhouse in Belgium. They were identified as *Brevibacillus brevis* or *Bacillus subtilis* by Biolog test and partial 16s rDNA sequence comparison. They not only inhibited the radial growth of the root-infecting fungi *Rhizoctonia solani* SX-6, *Pythium aphanidermatum* ZJP-1 and *Fusarium oxysporum* f.sp. *cucumerinum* ZJF-2 in vitro, but also exhibited strong nematicidal activity by killing the second stage larvae of *Meloidogyne javanica* to varying degrees in the greenhouse. The toxic principles of bacterium B7 that showed the highest juvenile mortality were partially characterized. The active factors were heat stability and resistance to extreme pH values. B7 used either as seed dressing or soil drench significantly reduced the nematode populations in the rhizosphere and enhanced the growth of mungbean plants over the controls in the presence or absence of *R. solani*.

Key words: Biocontrol, *Meloidogyne javanica*, Mungbean, Rhizobacteria, *Rhizoctonia solani*
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INTRODUCTION

Crops production in greenhouses is a method for economically maintaining a warm environment during cool seasons and to protect crops, especially vegetables, from unfavourable weather conditions. However, root-knot nematodes and soil borne fungi greatly affect plant growth and yield in greenhouses. Plant-growth promoting rhizobacteria (PGPR) have been identified as a biological control alternative to pesticide use for disease suppression without negative effects on the user, consumer or the environment (Johnsson *et al*., 1998). In our previous study, 4 bacteria with high potential for promoting plant-growth and suppression of soil-borne pathogens were screened from over 500 bacteria isolated from rhizosphere of vegetables in China (Soad *et al*., 2004). The present study was initiated to further determine the identity of the 4 rhizobacteria, investigate their potential for controlling root-knot nematode, in the presence or absence of *Rhizoctonia solani* in greenhouse vegetable production in Belgium, and to partially characterize the active nematicidal principles of a rhizobacterium B7.

MATERIALS AND METHODS

The pathogens and mungbean seed

The root-infecting fungi *Pythium aphanidermatum* ZJP-1 and *Fusarium oxysporum* f.sp. *cucumerinum* ZJF-2 were provided by the Institute of Biotechnology, Zhejiang University, China. *R. solani* SX-6 was obtained from the Laboratory of Phytopathology and Plant Protection, Katholieke Universiteit

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Leuven, Belgium. Root-knot nematode *M. javanica* eggs from tomato roots were placed in sterile water and incubated for 48 h at room temperature until they hatched. The hatched juveniles were collected in a beaker. Mungbean seeds used in the experiment were harvested from a cultivar "Haricots mungo" in China.

Identification of the rhizobacteria

The 4 rhizobacteria were maintained in Tryptic soy agar (Mew and Misra, 1994) for routine use and in 30% glycerol at −20 °C for long-term storage. The phenotypic identification was done using BIOLOG (Biolog Inc., California USA) according to the instructions of the manufacturer. After total DNA of the rhizobacteria were extracted by the CTAB method (Ausubel *et al*., 1992), the 16S rRNA gene was amplified as described by Han *et al*.(2002) using the forward (16F27: 5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse (16R1522: 5′-AAG GAG GTG ATC CAG CCG CA-3′) primers. Purification and sequencing of amplified 16S rDNAs were completed by Shanghai Bioasia Company. Sequence data were compared with those of the GenBank database.

In-vitro antibiosis test

Antifungal activity. The dual culture plate method was employed to examine the inhibition of rhizobacteria against root-infecting fungi. Bacterial isolates were streaked onto one side of the PDA (Potato Dextrose Agar) plate. On the other side of each Petri plate, an agar disc plug of 5 mm in diameter with the target fungus was inoculated. The plates were incubated at 28 °C and the inhibition zone (if any) was measured after one week. Only those isolates that produced a clear inhibition zone were considered effective (Siddiqui *et al*., 2001).

Nematicidal activity. For the preparation of cell-free culture filtrate, the bacteria were centrifuged twice at 12000 rpm for 20 min and pellets were discarded. To determine the effect of bacterial culture filtrate on *M. javanica* juveniles, 1.5 ml of the filtrate was transferred in a 10 ml vessel to which 1 ml juvenile suspension (30−35 juveniles/ml) was added. After 48 h, the number of dead juveniles was counted. The juveniles were considered dead when they did not move on probing with a fine needle. The active nematicidal principles of B7 that caused heavy juvenile mortality were partially characterized. Heat resistance, pH stability test and the solubility of the active principles were determined as described by Ali *et al*.(2002). In another test, the filtrate was frozed for 2 h at −70 °C to investigate the nematicidal activity.

In-vivo biocontrol trials

After preliminary tests with a number of different plants, we chose mungbean as the test plant because of its high germination rate (~98%), fast growth, convenient plant size, and susceptibility to both *R. solani* and *M. javanica*. Greenhouse trials were conducted in seedling trays with sterile soil (Asef B.V., Didam, the Netherlands). Mungbean seeds surface sterilized in 1% NaClO for 3 min were rinsed 3 times with sterile distilled water. For application of the bacteria as a soil drench, a 25-ml aqueous cell suspension (10^8 cfu/ml) was used. Soil drenched with 25-ml sterile water served as control. One week after seedling emergence, each pot was inoculated with 800 freshly hatched juveniles of *M. javanica*. Treatments and controls were replicated three times and the pots were arranged in randomised complete blocks. Eight weeks after the nematode inoculation, the number of the root-knot nematodes was recorded as described by Ali *et al*.(2002).

The nematicidal activities of B2 and B7 that caused the greatest reduction in nematode population were further evaluated in the presence of the fungi. For seed dressing, mungbean seed surface sterilized as described above were treated with an aqueous cell suspension of the bacteria to yield approximately 1.2×10^5 cfu/seed. Bacteria were applied as a soil drench was described earlier. Meanwhile, *R. solani* SX-6 was incubated on PDB (broth) for 4 d as shake culture at 28 °C. One week after seedling emergence, each pot was inoculated either by co-infection of 800 freshly hatched juveniles and 20 ml culture liquid of *R. solani* or *M. javanica* alone. The rest of procedure was the same as outlined above.

Statistical analysis

Data were subjected to one-way analysis of variance followed by least significant difference (LSD) test at P=0.05 and Duncan's multiple-range test by using DPS software (Version 5.0). Data on the bacterial populations was transformed to $\log_{10}(x+1)$ to avoid $\log_{10}0$ in the analysis.

RESULTS

Effect of rhizobacteria on inhibition of root-knot nematodes and fungal pathogens

All 4 rhizobacteria tested were antagonistic to *R. solani* SX-6 and *P. aphanidermatum* ZJP-1 in dual culture. Isolate B2 showed the largest inhibition zone against *R. solani* SX-6 and B7 exhibited inhibition of growth against all the three fungal pathogens tested. Culture filtrates of the rhizobacteria showed nematicidal effects, killing 62% to 70% the second stage juveniles of *M. javanica* (Table 1). The maximum juvenile mortality was recorded with isolates B7 and B2, followed by isolate B8 and B5 that resulted in 64% and 62% juvenile deaths, respectively.

Table 1 In vitro antifungal and nematicidal effects of selected rhizobacteria

Treatment	Zone of inhibition (mm)			Nematode mortality (%)
	ZJP-1	ZJF-2	SX-6	
B2	15.7a*	0.0b	19.5a	70.0a
B5	11.0b	0.0b	3.2d	62.0b
B7	17.0a	3.7a	8.0c	70.0a
B8	12.0b	0.0b	14.4b	64.0b
CK	–	–	–	15.0c

*In each column, means followed by the same letter are not significantly different at the 0.05 levels by Duncan's multiple-range test; refer to Table 1 in later Tables

Effect of the different treatments with filtrate of a rhizobacterium on mortality of *M. javanica*

Fig.1 shows that the culture filtrate of rhizobacterium B7 was alkaline resistant, and heat and cold stable. No significant difference was observed on juvenile mortality when the filtrate was boiled for 5 min or freezing at −70 °C. However, the filtrate resulted in significant reduction in juvenile mortality after acid treatment compared to the control. Among the three solvents used in the culture filtrate extraction, chloroform had no effect on the juvenile mortality while ethyl acetate and hexane showed significant reduction in the mortality at 48 h.

Effect of soil drench on the suppression of nematodes and growth promotion of mungbean

The four rhizobacteria applied as soil drench suppressed nematode population densities in soil to varying degree compared to that of CK1, the nematode control (Table 2). B7 and B2 caused the greatest

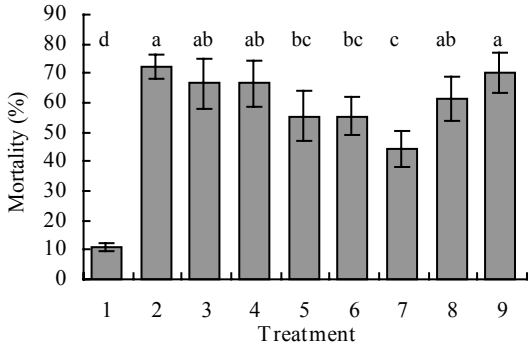

Fig.1 Effects of the cell-free culture filtrate of rhizobacterium B7 with different treatments on mortality of *M. javanica in vitro*

1: King B broth; 2: Boiling at 100 °C for 5 min; 3: Refrigeration at −70 °C for 2 h; 4: NaOH treatment at pH 12.0 for 30 min; 5: HCl treatment at pH 2.0 for 30 min; 6: Ethyl acetate extraction; 7: Hexane extraction; 8: Chloroform extraction; 9: Bacterial filtrate. Means on the bars followed by the same letter are not significantly different at the 0.05 levels by Duncan's multiple-range test

reduction (52.4% and 45.5%, respectively) in nematode population, followed by B5's and B8's 29.8% and 25.2%, respectively, when inoculated with nematode alone. B7 and B2 when inoculated with nematode and *R. solani* significantly reduced the nematode population in soil by 49.0% and 38.6%, respectively. In the presence of the fungal pathogen (CK3), the root-knot nematode population in the soil peaked. The data also showed that B7 significantly promoted the growth of mungbean plants inoculated either with nematode alone or with nematode and *R. solani*. However, B2 promoted the mungbean growth only in the absence of *R. solani*.

Effect of seed dressing on suppression of nematodes and growth promotion of the mungbean

Further studies were conducted to determine the effect of seed dressing of B2 and B7 against the root-knot nematode under greenhouse conditions. Strain B7, inoculated either with nematode alone or nematode and *R. solani*, significantly reduced the nematode populations by 56.7% and 43.6%, respectively in the soil, followed by B2 (52.4% and 36.1% reduction, respectively) compared to that of CK4, the sterile water controls (Table 3). Interaction between *R. solani* and *M. javanica* resulted in a significant increase of the nematode populations compared to that of the nematode inoculated alone. However, in the presence of *R. solani* SX-6, both rhizobacteria also

Table 2 Effect of soil drench with rhizobacteria on root-knot nematode populations in autoclaved soil[1] and growth of mungbean under greenhouse conditions inoculated either with nematode alone or with nematode and *R. solani*

Treatments	*NP*[5]	Height[6] (cm)	Stem (g/st[9])		Root (g/st)	
			FW[7]	*DW*[8]	*FW*	*DW*
B2+N[2]	427f	11.67a	0.375a	0.145ab	0.057c	0.031b
B5+N	550e	10.18bc	0.334b	0.137b	0.065ab	0.026c
B7+N	373g	10.25b	0.385a	0.151a	0.070a	0.036a
B8+N	586de	9.99bcd	0.315bc	0.114cd	0.040e	0.018e
B2+N+R[3]	733c	10.26b	0.333b	0.122c	0.050d	0.023cd
B7+N+R	608d	10.24b	0.383a	0.153a	0.060bc	0.032b
N (CK1)	783b	9.77de	0.295c	0.114cd	0.047de	0.021de
R (CK2)	0.00h	9.93cd	0.312bc	0.119cd	0.047de	0.024cd
N+R[4] (CK3)	1193a	9.53e	0.319bc	0.112d	0.046de	0.024cd
Sterile water (CK4)	0.00h	9.94cd	0.332b	0.138b	0.050d	0.027c

[1]The soil was sterilized in an autoclave at 121 °C for 60 min; [2]N: Inoculated with the root-knot nematode; [3]R: Inoculated with *R. solani*; [4]N+R: Inoculated with *R. solani* and the root-knot nematode; [5]*NP*: Nematode population in 250 cm^3 soil; [6]The average height of 4 seedlings retained per pot after germination ; [7]*FW*: Fresh weight; [8]*DW*: Dry weight; [9]g/st: Weight of a stick of mungbean

Table 3 Effect of seed dressing with selected rhizobacteria on root-knot nematode populations in autoclaved soil and on growth of mungbean plants under greenhouse conditions inoculated either with nematodes alone or with nematode and *R. solani*

Treatment	*NP*[4]	Height (cm)	Stem (g/st)		Root (g/st)	
			FW[5]	*DW*[6]	*FW*	*DW*
B2+N[1]	381d	7.31b	0.295cd	0.111bc	0.052cd	0.029b
B2+ N+R	672c	7.58b	0.319bc	0.113b	0.060bc	0.031b
B7+N	347d	8.48a	0.366a	0.123a	0.082a	0.036a
B7+ N+R	593c	7.43b	0.330b	0.114b	0.057c	0.024c
N (CK1)	801b	5.95d	0.252e	0.088d	0.030f	0.013e
R[2] (CK2)	0.00e	5.13f	0.196f	0.081d	0.031f	0.015e
N+R[3] (CK3)	1051a	5.65e	0.266de	0.105c	0.038e	0.017de
Sterile water (CK4)	0.00e	6.57c	0.325b	0.110bc	0.048d	0.020cd

[1]N: Inoculated with the root-knot nematode; [2]R: Inoculated with *R. solani*; [3]N+R: Inoculated with *R. solani* and the root-knot nematode; [4]*NP*: Nematode population in 250 cm^3 soil; [5]*FW*: Fresh weight; [6]*DW*: Dry weight

significantly suppressed the nematode population densities in soil. Strain B7 resulted in maximum plant height (29.1% increase), fresh or dry weight of stem (12.6% and 11.8% increase, respectively) and fresh or dry weight of root (70.8% and 80.0% increase, respectively) when inoculated with nematode alone. Less effect on the growth promotion was noted in the presence of *R. solani* SX-6. However, under these conditions, B2 still promoted growth of the mungbean roots.

Effect of the two application methods on populations of the rhizobacteria and *R. solani*

Total populations of the rhizobacteria in the rhizosphere were higher for soil drench in all the treatments compared to those of the seed dressing (Fig.2). As soil drench, rhizobacterium B2 and B7 inoculated with the nematode alone or with nematode and *R. solani* colonized in the rhizosphere well. The data also indicated that the 3 rhizobacteria, B2, B7 and B8, were not significantly affected in their colonization in the soil whether *R. solani* SX-6 was present or absent in the soil. Another set of data showed that the populations of *R. solani* SX-6 in the soil was not significantly increased in the presence of root-knot nematode, ranging from 1.82×10^5 to 3.98×10^5 cfu/g soils in the rhizosphere. It seemed that there was no significant difference between the two

application methods with respect to effect on populations of the fungal pathogen.

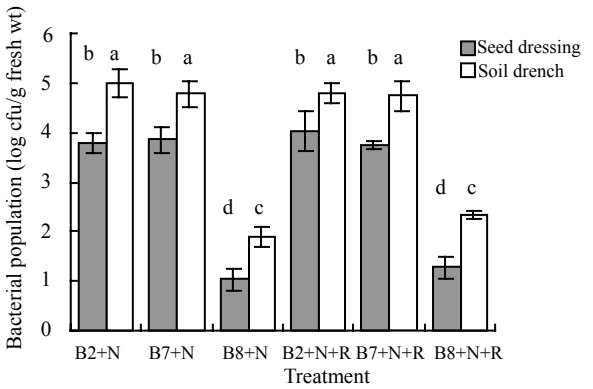

Fig.2 Bacterial colonization in the rhizosphere eight weeks after inoculation with nematode alone or nematode and *R. solani* under greenhouse conditions

Means on the bars followed by the same letter are not significantly different at the 0.05 levels by Duncan's multiple-range test. N: Inoculated with nematode alone; N+R: Inoculated with root knot nematode and *R. solani*; B2−8: Rhizobacterial isolates. Populations were counted by serial dilution plating

Identification of the rhizobacteria

BIOLOG identification showed indistinguishable reaction patterns for these rhizobacteria. They were identified as *Bacillus* sp. at the genus level when their profiles were compared with those of the Biolog GPdatabase (Version 4.01). A 16S rDNA fragment of approximately 1.5 kb was generated for each of the 4 rhizobacteria and more than 500 bp were sequenced. After comparison of these partial 16S rDNA sequences (EMBL accession Nos. AJ831420, AJ831421, AJ831422 and AJ831423) with those available from the public databases, B5, B7, B8, and B2 were further identified as *B. subtilis* (100% similarity), *B. subtilis* (99% similarity), *B. subtilis* (98% similarity) and *Brevibacillus brevis* (88% similarity), respectively.

DISCUSSION

Root-knot nematodes widely distributed in greenhouse crops causing substantial yield losses in Belgium and China are targets in biological control programmes based on the application of PGPR with antagonistic effect against these soil-borne pathogens. In our experiments rhizobacterium B7 showed high potential in suppressing the root-knot nematode that may be attributed to modification of root exudates, which interfere with the host finding processes of the nematodes or produces metabolites that are toxic to the nematodes. Chemotaxis towards exudate components has also been regarded as an important trait for root colonization (De Weert *et al*., 2002). The culture filtrates of B7 are heat stable and resistant to extreme pH values, which suggested that antibiotic rather than protein might be responsible for the nematicidal activity. On the other hand, growth-promoting effects of mungbean plant by B7 maybe the result from production of phytohormones that are reported to elongate stem and expand the root system (Davies, 1987). Kassab and Ali (1996) showed that interaction between the fungus and the nematodes resulted in the reduction of seed emergence and increase in both galling and nematode fecundity, which is consistent with our results that the population of *M. javanica* increased in the presence of *R. solani*. Simultaneously, the diseases development caused by soil-borne fungal pathogens was also stimulated by nematode (Shawadfy and Mousa, 1997). Our results also demonstrated this phenomenon, however, its severity varied with the bacterial strains. Whether the severity would be related to biotypes of the nematode is of interest for further study.

CONCLUSION

Our study indicated that the rhizobacteria isolated from China, identified as *Brebacillus* and *Bacillus* species, could survive in greenhouse conditions in Belgium. The selected isolates are antagonistic to root-knot nematodes and could be developed into a valuable crop management tool to reduce the deleterious impact of plant-parasitic nematodes on plant growth in the presence or absence of *R. solani*. Results from these studies should contribute to a better understanding of the complex interactions among root-knot nematodes, introduced rhizobacteria, soil-borne root-infecting fungi and host plant. Such information would be valuable for the isolation and characterization of the active nematicidal agents.

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