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ВЛИЯНИЕ НА РИЗОБАКТЕРИЯТА *BACILLUS SUBTILIS* ВЪРХУ ГАЛОВАТА НЕМАТОДА *MELOIDOGYNE ARENARIA*

МЕЛИКА МОХАМЕДОВА, ХАРИ САМАЛИЕВ

INFLUENCE OF THE RHIZOBACTERIUM *BACILLUS SUBTILIS* ON THE ROOT-KNOT NEMATODE *MELOIDOGYNE ARENARIA*

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Abstract

Two local strains of *Bacillus subtilis* (A1 and B1) were evaluated for their nematicidal effects on the root-knot nematode *Meloidogyne arenaria*. In laboratory experiment exposure of egg masses of *M. arenaria* to the bacterial strains resulted in reduced hatching of second stage juveniles (J2). J2 in contact with the strains of *B. subtilis* and their metabolites showed disorientation and convulsive movement. After their placement in fresh water many juveniles retrieved their normal movement. The highest mortality of J2 was observed at dosage of 10^8 and 10^9 cells ml^{-1} of A1 and B1 after 24 h exposure. In pot experiments strain A1 was ineffective but strain B1 prevented invasion of J2 of *M. arenaria* into tomato roots and affect nematode development in galls.

Key words: *Bacillus subtilis*, root-knot nematode, hatch, invasion, tomato

INTRODOCTION

Nematodes cause about 20.6% worldwide yield loss (Sasser, 1989). Root-knot nematodes (*Meloidogyne* spp.) are the most pathogenic nematodes which parasitize on vegetables growing on fields and in greenhouses in Bulgaria (Samaliev and Stoyanov, 2008). Plants infested with *Meloidogyne* spp. show stunting and typical symptoms of root-galling. The control of these pathogens includes mainly the use of pesticides. However, in many cases, high-toxic nematicides are required to reduce damage and increase yield (Sikora and Fernandez, 2005). Interest in biological control of nematodes has increased, fuelled by public concern over the use of chemicals in the environment in general and because of the need to find alternatives to fumigant and non-fumigant nematicides and overall improvement of IPM programmes (Moens et al., 2002; Gowen et al., 2005).

The rhizosphere of plants is a zone of intense microbial activity. In this zone nematodes encounters antagonism from rhizosphere microorganisms during

infection and in many cases this can lead to substantial disease control (Siddiqui and Mahmood, 2001). Rhizobacteria that exert beneficial effects on plant development and are capable of providing protection against nematode disease are referred to as plant growth-promoting rhizobacteria (PGPR; Kloepper et al., 1980). *Bacillus* is one of the important bacterial genera, which can suppress nematode invasion (Kloepper and Ryu, 2006). Gokte and Swarup (1988) reported that *Bacillus subtilis*, *Bacillus cereus* and *Bcillus pumilus* exhibited larvicidal activity against the second stage juveniles (J2) of *Meloidogyne incognita in vitro*. In pot experiment Gautam et al. (1995) observed reduction of *M. incognita* multiplication on tomato due to use of *B. subtilis* as seeds treatment. *B. firmus* caused paralysis and mortality of *Radopholus similis* and *M. incognita* juveniles *in vitro* (Mendoza et al., 2008) and decreased root-knot nematode infestation into tomato plants *in vivo* (Terefere et al., 2009).

In Bulgaria local strains of *Bacillus* spp. were isolated from various location. Some of them showed lethality to eggs and second stage juveniles of *Meloidogyne arenaria* Neal under laboratory conditions (Mohamedova and Samaliev, 2006).

The aim of the present experiments was to evaluate the nematicidal effects of two strains of *B. subtilis* (B1 and A1) on J2 of *M. arenaria in vitro*; the efficacy of these strains to suppress nematode invasion into tomato roots *in vivo* was investigated as well.

MATERIALS AND METHODS

Nematode culture and sterilization

The root-knot nematode *M. arenaria* was obtained from cultures derived from single egg masses maintained on tomato (*Lycopersicon esculentum* Mill., cv. Veolositi) in greenhouse at 24-26°C. Mature egg masses of *M. arenaria* were hand picked, using sterilized needle and forceps from heavily infested tomato roots. These egg masses were sterilized in streptomycin sulphate (0.1%) for 45 min (Sawhney and Webster, 1975) and rinsed in sterile distilled water (SDW) before use in experiments. J2 of *M. arenaria* were extracted from infested tomato roots. Galled roots with egg masses were washed free of soil, cut into small pieces, placed in 1.5% NaOCl and macerated in a blender (Hussey and Barker, 1973). The suspension was poured onto cotton-wool filter, incubated at 24-26°C and hatched J2 were collected every 24 h. J2 were sterilized in streptomycin sulphate (0.1%) for 15 min (Mountain, 1955) and rinsed in SDW before use in experiments.

Bacterium culture and identification

The strains B1 and A1of *B. subtilis* were cultivated at 28°C on tryptic soy broth (TSB). For inoculum production single colonies of the two strains were grown at 27°C for 48 h with shaking (150 rpm) in 250 ml Erlenmeyer flasks containing 100 ml of TSB. The bacterial suspensions were centrifuged at 2800 g for 20 min. The concentrated suspensions were diluted with sterile tap water to give the concentrations required for experiments. Bacterial concentrations were determined by using a spectrophotometer. Bacterial identity of both strains was determined by Fatty Acid Analysis (Microbial Identification System Inc., Delware, USA).

Effect of bacterium and its metabolites on the hatching of second stage juveniles of M. arenaria from egg masses

Bacterial suspensions of *B. subtilis* (strains B1 and A1) in concentrations of 0, 10^7, 10^8 and 10^9 cells/ml^{-1} were mixed with nutrient agar and cooled at 45ºC. Then the suspensions in different concentrations were transferred to 8.5 mm Petri dishes. Five egg masses containing approximately 750 eggs were inoculated in 0.2 ml SDW into the Petri dishes, which were sealed with Parafilm. The egg masses were exposed for 1, 3, 6 and 9 days at 25ºC. After exposure, the egg masses were washed and transferred to SDW and the number of J2 emerging from the egg masses over 10 days was counted. The experiment was conducted with four replication per treatment.

Effect of bacterium and its metabolites on second stage juveniles of M. arenaria

Bacterial suspensions of *B. subtilis* (strains B1 and A1) in concentrations of 0, 10^5, 10^6, 10^7, 10^8 and 10^9 cells/ml^{-1} were made up as in the previous experiment. 200 J2 in a drop of SDW (0.2) were inoculated onto the nutrient agar in Petri dishes and exposed for 3, 6, 12, 24, 48, 72 and 96 h at 25ºC. For the period of exposure the behaviour of the J2 was observed under binocular microscope, the number showing convulsive movements or paralysis was counted. After exposure, J2 were placed in SDW and observe for a further 3 days. After the completion of observation J2 were stained in the vital stain of bromothymol bleu. Those staining deep bleu were classified as dead. The experiment was conducted with four replication per treatment.

Effect of bacterium and its metabolites on the penetration of second stage juveniles of M. arenaria into tomato roots

Bacterial suspensions of *B. subtilis* (strains B1 and A1) containing of 0 and 10^8 cells/ml^{-1} were made up as in the previous experiment. 200 J2 in a drop of SDW (0.2) were inoculated onto the nutrient agar in Petri dishes and exposed for 12, 24, 48, 72 and 96 h at 25ºC. After exposure, J2 were placed in SDW for a further 3 h. Then J2 were inoculated in the rhizosphere of two weeks old tomato plants (cv. Velositi), growing in plastic pots (10 cm/diam.) in sterilized soil. The plants were maintained in a growth room at 25ºC with 16 h day/8 h night photoperiod. The plants were harvested 21 days after inoculation with J2, the roots were washed free of soil, stained in acid fuchsin (Bridge et al., 1982), macerated in a blender and the number of J2 into roots was counted. The experiment was conducted with four replication per treatment.

Statistical analysis

Data were analyzed by analysis of variance, using procedures of the SPSS programme. Least significant differences (LSD) were calculated at P=0.05 And Duncan`s multiple range test was employer to test for significant differences between treatments.

RESULTS AND DISCUSSION

Effect of bacterium and its metabolites on the hatching of second stage juveniles of M. arenaria from egg masses

The highest reduction in number of J2 (112 J) emerging from *M. arenaria* egg masses was observed at 10^9 cells/ml^{-1} of bacterial suspensions of B1 after 1 day

exposure (Table 1). At the same concentration and exposure of egg masses to bacterial suspension of A1 the number of hatching J2 was higher (140 J2) compared with those of B1. It was found out that hatching rate for *M. arenaria* J2 decreased as the bacterial concentration and time exposure for both strains increased. The least number of J2 emerging from egg masses treated with B1 and A1 was counted at 10^9 cells/ml^{-1} of bacterial suspensions after 9 days exposure. There was not significant difference in number of J2 emerging from egg masses at 10^8 and 10^9 cells/ml^{-1} of B1 and A1 bacterial suspensions after all evaluated exposure (Table 1).

Table 1

Hatch of *Meloidogyne arenaria* J2 from egg masses exposed to different concentration of *B. subtilis* (B1 and A1) during 1, 3, 6 and 9 exposure to the bacterial suspensions and after 10 days in sterile distilled water

Strains	Bacterial concentrations (cells/ml^{-1}) / Number of J2			
	0	10^7	10^8	10^9
		1 day		
Б1	690a	267b	121c	112c
A1	683a	288b	138c	140c
		3 days		
Б1	904a	196b	97c	82c
A1	919a	271b	112c	104c
		6 days		
Б1	963a	176b	75c	60c
A1	965a	199b	81c	68c
		9 days		
Б1	972a	157b	64c	51c
A1	965a	168b	86c	63c

Our results are consistent with this reported by Gokte and Swarup (1988) and Ali et al. (2002). The authors observed that the rhizobacteria *B. cereus*, *B. subtilis*, *B. pumilus* and *Pseudomonas aeruginosa* inhibited the hatch of *M. incognita* eggs. Similarly Mendoza et al. (2008) reported that after 3, 6 0r 9 days incubation in *B. firmus* culture filtrate, hatching rates of *M. incognita* juveniles were significantly lower compared to eggs incubated in the STB or sterile water. Rhizobacteria are known to produce toxic metabolites (Sikora and Hoffman-Hergarten, 1993) such as 2,4-diacetylphloroglucinol (Cronin et al., 1997) and abamektin (El-Nagti and Youssef, 2004). It is possible that the strains B1 and A1 of *B. subtilis* produce secondary metabolites which may be responsible for the effect observed in this experiment.

Effect of bacterium and its metabolites on second stage juveniles of M. arenaria

At concentration of 10^5 cells/ml^{-1} of bacterial suspensions of B1 and A1, J2 became disorientated and exhibited convulsive movements (Table 2). At 10^7 the effect of both strains was more marked i.e., there was increase in the percentage of J2 showing convulsive movements. Strain B1 had a faster initial effect on J2 than strain A1. At 10^7 cells/ml^{-1} of bacterial suspensions of B1 and A1 and

exposure for 48 h, the percentage of J2 exhibiting convulsive movements was 79,0% and 53,5%, respectively (Table 2). At concentration of 10^8 and 10^9 cells/ml^{-1} of bacterial suspensions of B1 and A1 all J2 were paralyzed after exposure for 72 and 96 h. Overall strain B1 affected J2 slightly more than strain A1. J2 became paralyzed almost immediately after contact with the bacterial suspension of B1. When J2 were placed in fresh SDW after their exposure to the bacterial suspensions of B1 and A1 it was observed that some of them retrieved theirs normal movement after exhibition convulsive movement or paralyzis but many remained paralyzed. The J2 were stained in bromothymol bleu to determinate how many of the paralyzed J2 were "dead". The percentage mortality of the exposed J2 was calculated and compared with the percentage paralyzed.

Table 2

The response of *Meloidogyne arenaria* J2 to exposure to different concentrations of bacterial suspensions of *B. subtilis* (B1 and A1), estimated as percentage showing convulsive movement

Exposure time, hours	Bacterial concentrations (cells/ml^{-1})									
	10^5		10^6		10^7		10^8		10^9	
	Б1	A1	Б1	A1	Б1	A1	Б1	A1	Б1	A1
3	9.6	2.0	17.1	4.0	22.7	8.0	28.0	10.7	30.2	13.3
6	19.5	6.5	24.8	8.6	30.2	13.7	38.2	17.5	39.4	20.2
12	23.3	15.8	32.5	19.8	36.3	26.8	44.2	33.7	45.7	34.8
24	52.6	23.7	68.5	37.2	79.0	48.4	0*	56.5	0*	59.7
48	53.2	30.4	70.7	47.5	79.8	53.5	0*	64.7	0*	69.8
72	54.6	49.2	72.5	69.7	0*	0*	0*	0*	0*	0*
92	56.2	53.1	74.3	71.2	0*	0*	0	0*	0*	0*

- All J2 paralyzed

Significant mortality of J2 treated with different concentrations of bacterial suspension of B1 was obtained after 3h exposure (Table 3). Bacterial suspension of A1 caused significant mortality of J2 at concentration of 10^6, 10^7, 10^8 and 10^9 cells/ml^{-1} after 6 h exposure(Table 4). The highest J2 mortality was 92.3% and 91.8% at 10^9 of B1 and A1 and 9 h exposure, respectively (Tables 3 and 4).

Table 3

Percentage mortality of second stage juveniles of *Meloidogyne arenaria* treated with different concentrations of bacterial suspension of *B. subtilis* (B1) at different exposures

Concentration of cells ($ml^{/-1}$)	Exposure (hours)						
	3	6	12	24	48	72	96
0	0a	0a	1.1a	1.1a	1.1a	1.2a	1.2a
10^5	7.4b	18.3b	28.1b	58.0b	59.0b	59.0b	59.5b
10^6	15.1b	21.8b	36.1b	76.6b	75.2b	75.2b	76.0b
10^7	20.2b	27.8b	39.7b	83.2b	83.3b	83.3b	83.0b
10^8	22.8cb	31.8b	44.0cb	87.3b	91.2b	92.0cb	92.1cb
10^9	22.3cb	33.36b	47.0cb	90.0cb	92.0cb	92.0cb	92.3cb

The results indicate that the rhizobacterium *B. subtilis* (B1 and A1) and its metabolites at lower concentrations of the bacterial suspensions had a nematostatic effect on *M. arenaria* J2. In these cases J2 showed convulsive movements and temporary paralyzis. At concentrations of 10^8 and 10^9 cells/ml^{-1} *B. subtilis* (B1 and A1) demonstrated namatocidal activity causing mortality of J2. The behavioural response of *M. arenaria* J2 to *B. subtilis* (B1 and A1) and its metabolites has many similarities to the response of *M. javanica* J2 to *P. oryzihabitans* (Samaliev et al., 2000) and to the response of *M. incognita* to *B. firmus* (Mendoza et al., 2008).

Table 4

Percentage mortality of second stage juveniles of *Meloidogyne arenaria* treated with different concentrations of bacterial suspension of *B. subtilis* (A1) at different exposures

Concentration of cells (ml$^{/-1}$)	Exposure (hours)						
	3	6	12	24	48	72	96
0	0a	0.1a	1.7a	1.7a	1.7a	1.7a	1.7a
10^5	0a	6.7a	22.5b	36.3b	36.5b	60.7b	60.8b
10^6	0.7a	8.5ab	25.0b	42.7b	47.6b	74.0b	73.8b
10^7	2.8a	7.7ab	30.0b	50.0b	53.1b	82.2b	82.7b
10^8	5.3a	15.0b	30.5b	51.6b	59.00b	88.0cb	88.0b
10^9	6.3a	16.5b	32.5b	54.7cb	61.00b	92.0cb	91.8cb

Effect of bacterium and its metabolites on the penetration of second stage juveniles of M. arenaria into tomato roots

Strain B1 containing 10^8 cells/ml^{-1} significantly reduced the number of J2 entering tomato roots, the effect being more marked after 72 and 96 exposure of J2 to the bacterial suspension (Fig.1).

The number of J2 entering tomato roots ranged from 11,3 to 7,3 after different exposures of J2 to B1. In roots of untreated plants the number of J2 ranged from 33 to 36. Strain A1 had no effect on the invasion of tomato roots by J2 and the number of penetrated J2 was the same as in untreated roots (Fig.1).

In this experiment bacterial suspension of the rhizobacterim *B. subtilis* (B1) reduced the invasion of *M. arenaria* into tomato roots. In addition it was noted that some of the developing root galls had no nematodes indicating that the developing juveniles may have disintegrated (data are not presented). The rhizobacteria *P. putida* and *P. alcaligenes* (Siddiqui and Akhtar, 2009) and *B. firmus* (Terefe et al., 2009) had adverse effects on penetration of *M. incognita* into plant roots. The authors also observed reduction of the nematode development into roots.

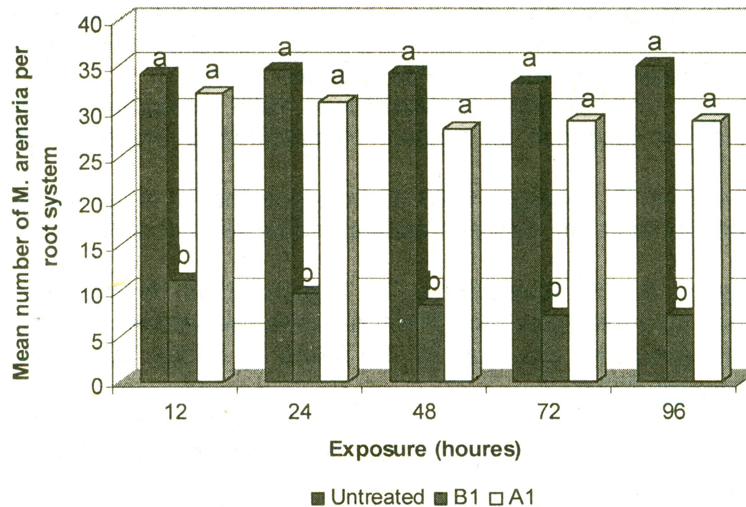

Fig.1. Number of *Meloidogyne arenaria* in tomato roots 21 days after inoculation with 200 J2, following different periods of juveniles exposure to 20 ml suspensions containing 10^8 cells/ml^{-1} of *B. subtilis* (B1 and A1)

CONCLUSONS

The results presented here indicate that both B1 and A1 strains of *B. subtilis* have nematicidal effect against *M. arenaria in vitro*. The efficacy of the strains depends on the concentration of the bacterial suspension and time exposure.

Only the strain B1 decreased penetration of *M. arenaria* into tomato roots. Strain A1 was ineffective in the pot experiment.

The rhizobacterium *B. subtilis* (B1) may be used for incorporation into IPM programmes against root-knot nematodes. More studies on the development of *Meloidogyne* spp. into plant roots and additional evaluation under greenhouse conditions are required to confirm this.

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