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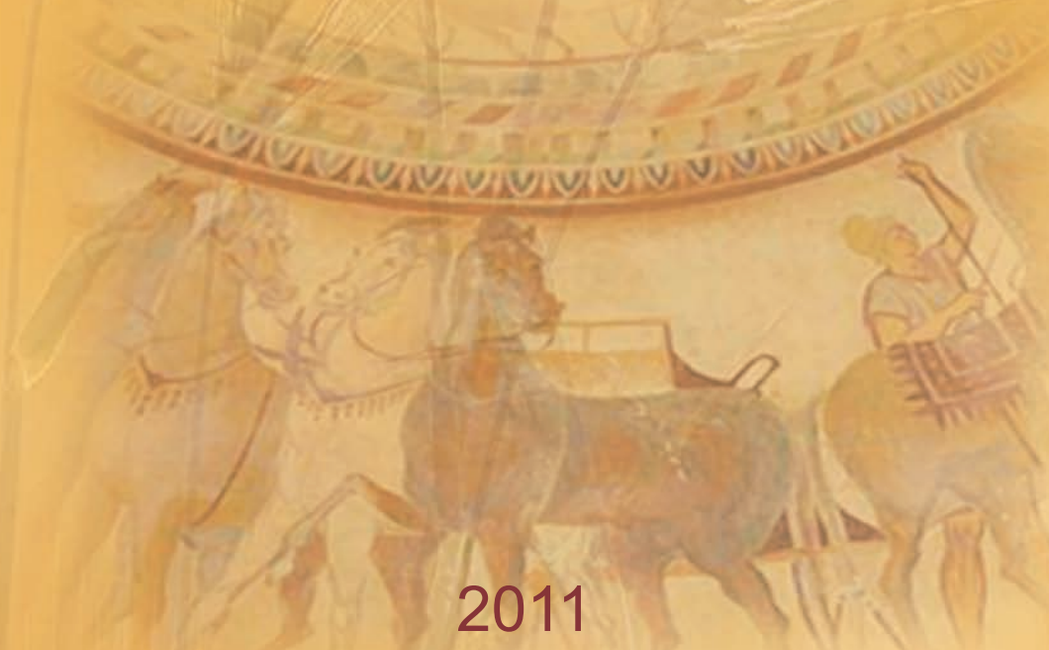

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Effect of the rhizobacterium *Bacillus subtilis* on the development of the root-knot nematode *Meloidogyne arenaria* at different temperatures

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Abstract. *Growth room experiment was carried out to investigate the effect of different temperatures on the efficacy of the rhizobacterium Bacillus subtilis on the development of the root-knot nematode Meloidogyne arenaria. Potato plants (Solanum tuberosum L.) were inoculated with M. arenaria second-stage juveniles. At 7th, 14th and 21th days after nematode inoculation the rizosphere of the plants were treated with bacterial suspensions of the two local strains of B. subtilis (A1 and B1). The potato plants were grown in growth room at 16±1, 20±1, 24±1, 28±1 and 32±1ºC. The rate of M. arenaria development in potato roots treated with B. subtilis (strains A1 and B1) was lower than that in the untreated plant roots. The highest efficacy of the bacterium on the development of M. arenaria was observed at 24-32ºC.*

Keywords: root-knot nematode, *Bacillus subtilis*, temperature influence, development, biological control

Introduction

Nematodes cause about 20.6% worldwide yield loss (Sasser, 1989). Root-knot nematodes (*Meloidogyne* sp.) are serious plant pathogens which parasitize on vegetables growing on fields and in greenhouses in Bulgaria (Samaliev and Stoyanov, 2008). 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). Recently interest in biological control of nematodes has increased, fuelled by public concern over the use of chemicals in the environment (Moens et al., 2004; Gowen et al., 2005).

Bacillus is one of the important bacterial genera, which can suppress nematode invasion (Kloepper and Ryu, 2006). Gokte and Swarup (1988) reported that *B. subtilis*, *B. cereus* and *B. 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. thuriengiensis* was reported as a potential control agents to many plant parasitic nematodes, including *Meloidogyne* sp. (Borgonie et al., 1996; Marroquin et al., 2000; Khyami-Horani and Al-Banna, 2006). In our previous studies some local strains of the rhizobacterium *B. subtilis* caused mortality of *M. arenaria* J2 in laboratory experiments (Mohamedova and Samaliev, 2006) and prevented invasion of J2 into tomato roots in pot experiments at 25ºC (Mohamedova and Samaliev, 2010).One of the factors limiting the effect of the rhizobacteria on root-knot nematodes is the temperature. Becker et al. (1992) reported the activity of *B. thuringiensis* var. *israelensis* towards different plant pathogens did not change significantly between 19 and 33°C. However, the effect of *Pseudomonas oryzihabitans* on *Globodera rostochiensis* hatch and J2 is highest between 21 and 25°C and decreasing below 20°C (Andreoglou and Gowen, 2000).The objective of this evaluation was to determine the effect of the rhizobacterium *B. subtilis* on *M. arenaria* development at different temperatures.

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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. Velositi) in greenhouse at 24-26ºC. 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 24-26ºC 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 A1 of *B. subtilis* were cultivated at 28ºC on tryptic soy broth (TSB). For inoculum production single colonies of the two strains were grown at 27ºC 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 Bacillus subtilis (strains A1 and B1) on the development of Meloidogyne arenaria at different temperatures

Sterile potato tubers (cv. Nadezda) with one sprout were planted in closed plastic containers (4.5 cm high, 10 cm in diameter) in 200 g of sterilized soil (3:1 loam/sand mixture), at 40% moisture-

level. This technique was modified by Phillips et al. (1980). The containers were stored in the dark at a constant temperature of 211C for 2 weeks. Two-week-old potato plants were inoculated with 200 J2 of *M. arenaria* and placed in an incubators, in dark, at 161C, 201C, 241C, 281C and 321C. After 7 days the plants were uprooted, washed free of soil and replanted in sterilized soil in new containers. At 7, 14 and 21 days after J2 inoculation, 20 ml of a bacterial suspensions containing 10^8 cells/ml of *B. subtilis* (strains A1 and B1) were applied to the soil around the roots. Four untreated plants received 20 ml of sterile distilled water and served as control. At each treatment 4 plants (fixed) were harvested to determine the stage of nematode development when the bacterium was applied. After each treatment the plants were placed back in the incubators. At 83, 51, 42, 33 and 36 days after J2 inoculation at 161C, 201C, 241C, 281C and 321C, respectively, the roots were collected, washed free of soil, stained in acid fuchsin (Bridge et al., 1982), macerated in a blender and the number of different stages of the nematode determined. The experiment was conducted with four replication per treatment.

Statistical analysis

All statistical analysis was performed using procedures of the SPSS programme, significance being determined at P=0.05. Because most of the data were counts out of a total, e.g. nematodes in roots after plant inoculation with 200 J2, most of the analysis that was done was logistic regression based on binomial data. However, some data were not analysed, because they were extreme (e.g. almost 0 and 100%) and the differences were obvious.

Results and discussion

The effect of *B. subtilis* (strains A1 and B1) on the development and fecundity of *M. arenaria* in potato roots was different at different temperatures. The influence of the strains was more pronounced the closer the bacterium was applied after J2 inoculation and the higher the temperature was. At 28±1ºC and 7 days after J2 inoculation strain B1 caused the highest inhibition of nematode development into plant roots. At this temperature only 25% of developed females had egg masses (Table 4). The effect of the strain was less marked at 24±1ºC and 32±1ºC. However, the percentage of females with egg masses in treated plants was significantly lower than this one into untreated roots, respectively, 37.5% and 41.7% (Tables 3 and 5).

After 14 days exposure and temperatures of 24±1ºC, 28±1ºC and 32±1ºC strain B1 significantly delayed egg masses formation compared to the untreated plants. The percentage of developed females with egg masses was respectively, 46.7%, 50.0% and 63.2% (Tables 3, 4 and 5). The number of eggs per egg masses was significantly lower than in the untreated plants, when the potato plants treated with the strain B1 up to 14 days after J2 inoculation at all investigated temperatures, and even after 21 days at 24±1ºC, 28±1ºC and 32±1ºC (Tables 3, 4 and 5).

Table 1. Effect of *B. subtilis* (A1 and B1) on the different stages of development of *M. arenaria* in potato roots 83 days after inoculation with 200 J2 at 16±1°C. Percentages were calculated as % of the nematodes counted in the roots

Strains	Days of bacteria treatment after J2 inoculation	Treatments	Developmental stages of *M. arenaria*				Eggs/egg mass[1]
			J2 (%)[2]	J3 (%)[2]	J4 (%)[2]	Females with egg masses (%)[2]	
A1	7		12.5(±4.7)	13.3(±4.6)	74.2(±4.1)	91.7(±5.6)	132
		Fixed	92.3	7.6	0	-	-
	14		6.5(±3.9)	14.4(±4.0)	79.1(±5.2)	100(±0.0)	154
		Fixed	37.7	62.3	0	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	165
		Fixed	10.2	31.1	58.7	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	168
	s.e.d						14.3
B1	7		24.6(±4.8)	23.3(±4.7)	52.1(±5.4)	85.0(±6.1)	138
		Fixed	96.7	3.3	0	-	-
	14		32.0(±6.2)	18.2(±5.3)	49.8(±5.1)	95.5(±6.4)	155
		Fixed	44.5	55.5	0	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	174
		Fixed	6.4	32.7	60.9	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	178
	s.e.d						17.8

[1] Data were analysed by analysis of variance. Summary statistics are mean values and the standard error of the difference.
[2] Data were analysed using logistic regression analysis. Summary statistics are mean counts (± standard error).
[3] Fixed plants were not analysed.

Table 2. Effect of *B. subtilis* (A1 and B1) on the different stages of development of *M. arenaria* in potato roots 51 days after inoculation with 200 J2 at 20±1°C. Percentages were calculated as % of the nematodes counted in the roots

Strains	Days of bacteria treatment after J2 inoculation	Treatments	Developmental stages of *M. arenaria*				Eggs/egg mass[1]
			J2 (%)[2]	J3 (%)[2]	J4 (%)[2]	Females with egg masses (%)[2]	
A1	7		10.7(±6.8)	11.3(±4.4)	78.0(±5.7)	94.7(±3.9)	128
		Fixed	89.8	10.2	0	-	-
	14		3.9(±4.0)	11.5(±4.8)	84.6(±6.1)	95.5(±4.7)	160
		Fixed	28.6	71.4	0	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	164
		Fixed	1.3	19.5	79.2	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	176
	s.e.d						15.8
B1	7		15.7(±4.8)	16.5(±5.2)	67.8(±6.1)	72.7(±6.7)	119
		Fixed	92.2	7.8	0	-	-
	14		12.8(±6.0)	14.8(±6.6)	72.4(±6.5)	83.3(±5.8)	147
		Fixed	24.5	75.5	0	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	160
		Fixed	1.1	17.2	81.7	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	171
	s.e.d						19.4

Table 3. Effect of *B. subtilis* (A1 and B1) on the different stages of development of *M. arenaria* in potato roots 42 days after inoculation with 200 J2 at 24±1°C. Percentages were calculated as % of the nematodes counted in the roots

Strains	Days of bacteria treatment after J2 inoculation	Treatments	Developmental stages of *M. arenaria*				Eggs/egg mass[1]
			J2 (%)[2]	J3 (%)[2]	J4 (%)[2]	Females with egg masses (%)[2]	
A1	7		5.8(±5.9)	11.4(±3.6)	82.8(±5.7)	37.5(±6.5)	81
		Fixed	87.3	12.7	0	-	-
	14		0(±0.0)	6.8(±4.9)	93.2(±3.8)	46.7(±6.1)	93
		Fixed	10.7	86.2	3.1	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	50.0(±5.2)	125
		Fixed	0	8.5	91.5	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	183
	s.e.d						17.1
B1	7		6.8(±3.9)	7.6(±4.1)	85.6(±3.8)	68.2(±5.5)	122
		Fixed	81.2	18.8	0	-	-
	14		1.1(±3.4)	3.5(±6.7)	95.4(±3.3)	81.5(±4.9)	165
		Fixed	12.2	82.0	5.8	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	174
		Fixed	2.8	7.8	89.4	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	189
	s.e.d						18.5

Table 4. Effect of *B. subtilis* (A1 and B1) on the different stages of development of *M. arenaria* in potato roots 33 days after inoculation with 200 J2 at 28±1°C. Percentages were calculated as % of the nematodes counted in the roots

Strains	Days of bacteria treatment after J2 inoculation	Treatments	Developmental stages of *M. arenaria*				Eggs/egg mass[1]
			J2 (%)[2]	J3 (%)[2]	J4 (%)[2]	Females with egg masses (%)[2]	
A1	7		4.6(±5.3)	15.4(±6.7)	80.0(±4.1)	25.0(±6.4)	84
		Fixed	84.2	15.8	0	-	-
	14		0(±0.0)	4.8(±6.9)	95.2(±5.0)	50.0(±5.7)	99
		Fixed	8.9	87.4	3.7	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	47.1(±6.0)	126
		Fixed	0	5.1	94.9	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	186
	s.e.d						25.5
B1	7		3.3(±5.8)	11.7(±4.7)	85.0(±5.1)	72.2(±4.6)	124
		Fixed	80.2	19.8	0	-	-
	14		0(±0.0)	3.0(±3.7)	97.0(±5.0)	88.0(±3.9)	166
		Fixed	9.6	85.2	5.2	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	175
		Fixed	0	7.3	92.7	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	190
	s.e.d						17.2

Table 5. Effect of *B. subtilis* (A1 and B1) on the different stages of development of *M. arenaria* in potato roots 36 days after inoculation with 200 J2 at 32±1°C. Percentages were calculated as % of the nematodes counted in the roots

Strains	Days of bacteria treatment after J2 inoculation	Treatments	Developmental stages of *M. arenaria*				Eggs/egg mass[1]
			J2 (%)[2]	J3 (%)[2]	J4 (%)[2]	Females with egg masses (%)[2]	
A1	7		1.9(±3.5)	7.2(±5.8)	90.9(±5.6)	41.7(±6.8)	92
		Fixed	79.2	20.8	0	-	-
	14		0(±0.0)	6.1(±6.0)	93.9(±4.7)	63.2(±6.3)	103
		Fixed	3.4	89.8	6.8	-	-
	21		0(±0.0)	0(±0.0)	100(±0.0)	80.6(±6.0)	142
		Fixed	0	3.2	96.8	-	-
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	180
	s.e.d						22.4
B1	7		1.3(±4.2)	4.8(±3.7)	93.9(±3.8)	88.0(±4.3)	120
		Fixed	77.7	22.3	0		
	14		0(±0.0)	1.6(±3.0)	98.4(±5.1)	93.3(±4.4)	155
		Fixed	4.4	87.1	8.5		
	21		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	168
		Fixed	0	4.1	95.9		
	Control		0(±0.0)	0(±0.0)	100(±0.0)	100(±0.0)	188
	s.e.d						19.7

B. subtilis (strain A1) showed lower effect on the development and reproduction of *M. arenaria* into plant roots than the strain B1 (Tables 1, 2, 3, 4 and 5). Significantly lower percentage of females with egg masses into roots treated with the strain A1 compared to untreated plants was observed only after one day exposure and temperatures of 24±1ºC and 28±1ºC, respectively, 68.2% and 72.2% (Tables 3 and 4).

This study demonstrates that the bacterium *B. subtilis* (strain B1) inhibits *M. arenaria* development and fecundity into potato roots. In the bioassays the effect of the strain depended on the temperature. The best effectiveness of the B1 bacterial suspension was observed at temperatures from 24C to 28C and in the cases when the plant roots contained J2 and J3 at the time of strain application. The nematicidal effect appears quickly at 24±1ºC, 28±1ºC and 32±1ºC and slowly at 20±1ºC. There was no effect of B1 at 16±1ºC. Our results are consistent with this reported by Andreoglou and Gowen (2000). The authors observed the highest effect of *P. oryzihabitans* against *G. rostohiensis* at temeratures from 22ºC to 26ºC. Hackenberg and Sikora (1994) reported that the rhizobacterium *Agrobacterium radiobacter* reduced the development of *G. pallida* significantly up to 70%, both at 20ºC and 25ºC.

B. subtilis (strain A) caused 90% mortality of *M. arenria* J2 and significantly decreased egg hatching *in vitro* at temperatures between 24ºC and 30ºC. However its effect *in vivo* was insignificant. This probably due to delayed and less bacterial colonization around plant roots or restricted possibility to produce secondary metabolites which are released into the soil and interfere with *M. arenaria* at they inhibit its development. Similarly Siddiqui and Mahmood (1992) reported that two strains of *B. licheniformis* with the same effect against *M. incognita in vitro* showed different effectiveness *in vivo*. The authors explained the results with different levels of root colonization of the strains.

Conclusion

Our study indicates that the rhizobacterium *B. subtilis* (strain B1) is nematicidal *in vivo* at temperatures between 20ºC and 32ºC. Below and above these temperatures the effect of the strain on the development and reproduction of *M. arenaria* in plant roots is insignificant. Usually planting of the vegetables (tomato) in plastic greenhouses occurs at the beginning of March, when the invasive J2 of *M. arenaria* infect plant roots at soil temperature of below 16ºC, i. e. lower that effective ones for *B. subtilis* (strain B1) used in this experiment. However, egg laying in the egg masses starts at soil temperatures of over 17ºC In this early periods of plant growth is recommend to combine the application of the strain B1 with reduced dosage of nonfumigant nematocides to achieve successful control of *M. arenaria*.

After all the selection of flexible strains effective at lower temperatures could be an important alternative in the development of *B. subtilis* as a biological control agent.

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Instruction for authors

Preparation of papers

Papers shall be submitted at the editorial office typed on standard typing pages (A4, 30 lines per page, 62 characters per line). The editors recommend up to 15 pages for full research paper (including abstract references, tables, figures and other appendices)

The manuscript should be structured as follows: Title, Names of authors and affiliation address, Abstract, List of keywords, Introduction, Material and methods, Results, Discussion, Conclusion, Acknowledgements (if any), References, Tables, Figures.

The title needs to be as concise and informative about the nature of research. It should be written with small letter /bold, 14/ without any abbreviations.

Names and affiliation of authors

The names of the authors should be presented from the initials of first names followed by the family names. The complete address and name of the institution should be stated next. The affiliation of authors are designated by different signs. For the author who is going to be corresponding by the editorial board and readers, an E-mail address and telephone number should be presented as footnote on the first page. Corresponding author is indicated with *.

Abstract should be not more than 350 words. It should be clearly stated what new findings have been made in the course of research. Abbreviations and references to authors are inadmissible in the summary. It should be understandable without having read the paper and should be in one paragraph.

Keywords: Up to maximum of 5 keywords should be selected not repeating the title but giving the essence of study.

The introduction must answer the following questions: What is known and what is new on the studied issue? What necessitated the research problem, described in the paper? What is your hypothesis and goal ?

Material and methods: The objects of research, organization of experiments, chemical analyses, statistical and other methods and conditions applied for the experiments should be described in detail. A criterion of sufficient information is to be possible for others to repeat the experiment in order to verify results.

Results are presented in understandable tables and figures, accompanied by the statistical parameters needed for the evaluation. Data from tables and figures should not be repeated in the text.

Tables should be as simple and as few as possible. Each table should have its own explanatory title and to be typed on a separate page. They should be outside the main body of the text and an indication should be given where it should be inserted.

Figures should be sharp with good contrast and rendition. Graphic materials should be preferred. Photographs to be appropriate for printing. Illustrations are supplied in colour as an exception after special agreement with the editorial board and possible payment of extra costs. The figures are to be each in a single file and their location should be given within the text.

Discussion: The objective of this section is to indicate the scientific significance of the study. By comparing the results and conclusions of other scientists the contribution of the study for expanding or modifying existing knowledge is pointed out clearly and convincingly to the reader.

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Book chapter or conference proceedings: Author(s) surname and initials, year. Title. In: Title of the book or of the proceedings followed by the editor(s), volume, pages. Name of publisher, place of publication. Example:

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Thesis:

Penkov D, 2008. Estimation of metabolic energy and true digestibility of amino acids of some feeds in experiments with muscus duck (Carina moshata, L). Thesis for DSc. Agrarian University, Plovdiv, 314 pp.

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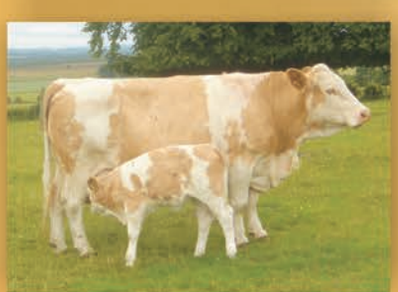

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