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Effects of rhizobacteria on *Meloidogyne javanica* infection on eggplants

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The effect of two rhizobacteria, *Pantoea agglomerans* and *Bacillus subtilis* on parasitism of the root-knot nematodes, *Meloidogyne javanica* on eggplants was evaluated in laboratory and greenhouse experiments. The impacts of the bacteria application as seed treatment, root dipping, and soil drench on early nematode penetration into plant roots, as well as, on eggplant growth were tested. The number of penetrated second stage juveniles of *M. javanica* was significantly reduced after *P. agglomerans* and *B. subtilis* application at concentration of 10^8 CFU ml^{-1}. *P. agglomerans* increased the eggplant fresh shoot and root weight following seed treatment. In the greenhouse experiment the bacteria were applied alone and in combination with a carbamate nematicide, Oxamyl (Vydate®). *P. agglomerans* was able to suppress *M. javanica* development into plant roots through a first half of eggplant cropping season. At the end of cropping season *B. subtilis* significantly reduced root gall index and number of nematode juveniles in soil and roots. This bacterium applied in combination with Oxamyl was the most efficient against *M. javanica* reproduction. Thereby, *B. subtilis* might be considered as a good candidate for biological or integrated control of the root knot nematodes.

Key words: *Bacillus subtilis*, *Pantoea agglomerans*, biological control, plant growth, gall index, root knot nematode.

INTRODUCTION

Root-knot nematodes are one of the main problems for protected crops, especially in south-eastern countries of Europe where climatic conditions favour their development. Species of *Meloidogyne* have a wide range of plant hosts and cause severe damage to vegetable crops. Crop losses due to *Meloidogyne* exceed 32% on tomato, 30% on melon and 20% on eggplant (Netscher and Sikora, 1990). Eggplant (*Solanum melongena* L.) is a traditional vegetable crop grown in Bulgaria. One of the most frequently found root-knot nematode species on eggplant is *Meloidogyne javanica*, present alone or in combination with other *Meloidogyne* species. In Bulgaria annual crop losses due to *M. javanica* largely depend on vegetable crops, their resistance to the nematode and the methods of control that have been used (Samaliev and Baicheva, 2010; Masheva et al., 2016). Present strategies for nematode management involved some cultural practices such as crop rotations and resistant

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varieties. Crop rotation is widely use but is not effective when applied for control of polyphagous pests. Due to the lack of resistance genes in many vegetables, resistant varieties are not readily available (Dias-Arieira et al., 2012). During the last ten years, a series of field studies employed throughout the world demonstrate that fumigant and non-fumigant nematicides are still efficient for the control of the root-knot nematodes (Giannakou and Anastasiadis, 2005; Choudhary et al., 2006; Hadian et al., 2011). However, the nematicides are usually toxic to highly toxic and there is much concern about their residual effects on the environment and human health. For this reason the interest in biological control as an alternative to chemical control of *Meloidogyne* has increased.

Bacteria associated with roots and rhizosphere of many plant species have been extensively tested for the control of various soil borne pathogens (Haseeb et al., 2006; Vasebi et al., 2015) including plant parasitic nematodes (Kluepfel et al., 1993; Insunza et al., 2002; Aballay et al., 2013). Currently, *Bacillus spp.* and *Pseudomonas spp.* are the most studied bacteria as biological control agents of root-knot nematodes (Hallmann et al., 2009). Other rhizosphere bacteria expressing antagonistic potential against *Meloidogyne* include, *Burkholderia, Paenibacillus*, *Pantoea* and *Seratia* (Duponnois et al., 1999; Oliveira et al., 2007; Abd-Elgawad and Kabeil, 2010). Control of nematodes by rhizosphere bacteria is achieved by mechanisms such as: direct antagonism through the production of secondary metabolites; interference with plant-nematode recognition; competition for nutrients; plant growth promotion; and induced systemic resistance (Tian et al., 2007). The low level in consistency of control of nematodes by many bacteria under field conditions is most likely due to the variability of the soil properties (physical and chemical), microbial activity in the soil as well as due to other environmental factors (Kerry, 2000). Thus, achieving efficient and consistent performance of biocontrol agents requires a more knowledge of effective screening techniques, and development of appropriate formulation and application techniques. The objectives of the present studies were: (i) to determine the effect of two rhizobacteria on early root penetration and gall formation of *M. javanica* into eggplant roots; *(*ii) to observe the influence of the bacteria on the plant growth; to examine the effect of bacteria alone or in combination with an organophosphate nematicide under greenhouse condition.

MATERIALS AND METHODS

Strains of rhizobacteria

Rhizobacteria were isolated in Bulgaria from rhizosphere of tomato plants. Two selected strains of the rhizobacteria, *Pantoea agglomerans* and *Bacillus subtilis* (collection of LMBT, Agricultural University-Plovdiv) which can significantly reduce incidence of galls of nematodes on tomato were used in this study.

Bacterium culture and identification

The strains of *P. agglomerans* and *B. subtilis* were grown in the dark for 48 h at 24°C on tryptic soy broth agar (TSBA). For inoculum production a loop of the bacteria was transferred into 100 ml of TSB and allowed to multiply for 48 h on a rotary shaker (160 rpm) at the same temperature. Bacterial suspensions were centrifuged at 4000 rpm for 20 min, and the bacterial pellet was resuspended in sterile ¼ strength Ringer's solution (Merck). The bacterial suspension was adjusted to a final concentration of 10^8 CFU ml^{-1} by dilution with Ringer's solution. The bacterial strains identifications were determined by FAME Analysis, following by BIOLOG Analysis for *P. agglomerans*.

Nematode culture and sterilization

M. javanica was obtained from cultures derived from single egg masses maintained on tomato (*Solanum lycopersicum* L., cv. Ideal) in a greenhouse at 24 to 26°C. Mature egg masses of *M. javanica* were hand-picked, using a sterilized needle and forceps from heavily infested tomato roots. These egg masses were sterilized in streptomycin sulphate (0.1%) for 45 min and rinsed in sterile distilled water (SDW) before being used as inoculum in experiments. Second stage juveniles (J2) of *M. javanica* were extracted from infested tomato roots following the procedure described by Stetina et al. (1997). Galled roots with egg masses were washed free of soil, cut into small pieces, placed in 1.5% NaOCl and homogenized at 8000 rpm (T 18 digital ULTRA-TURRAX) (Hussey and Barker, 1973). The suspension was poured onto cotton-wool filter, incubated at 24 to 26°C and hatched J2 were collected every 24 h. The second stage juveniles were also sterilized in streptomycin sulphate (0.1%) for 15 min and rinsed in SDW before being used in experiments.

Effect of rhizobacteria on the penetration rate of *M. javanica* juveniles

The bacteria were applied as follows:

1. Seed treatment: The eggplant seeds, cv. Lych were soaked in the suspensions of *P. agglomerans* and *B. subtilis* for 20 min and then planted into pots containing 500 ml soil/sand mixture (1:1, v/v). Each pot received 1 seed. After four weeks, the plants were inoculated with 2500 J2 of *M. javanica* per pot. The inoculation of nematodes was carried out by drenching 5 ml suspension of J2 in distilled water into the potted soil arround the roots. The control treatments received 5 ml distilled water;
2. Root dipping: Roots of three-week-old eggplants were dipped for 20 min into the bacterial suspensions and then planted into pots containing 500 ml soil/sand mixture (1:1, v/v). After a week, the plants were inoculated with 2500 J2 of *M. javanica* per pot.
3. Soil drench: Ten milliliter of bacterial suspensions was pipetted onto the soil surface around three-week-old eggplants. Plants were inoculated with 2500 J2 of *M. javanica* one week after bacteria application. The inoculation of nematodes was carried out by drenching 5 ml suspension of J2 in distilled water into the potted soil arround the roots. The control treatments received 5 ml distilled water. Plants were kept in a growth room with following conditions, 28±1°C temperature, 70±5% relative humidity (RH) and 16:8 (L:D) photoperiod. All the experiments was replicated eight times, end terminated two weeks after nematode inoculation.

At the end of the experiments eggplant fresh shoot and root weight was measured and the penetration rate of *M. javanica* was recorded. Penetrated J2 of *M. javanica* into eggplant roots was determined after staining the roots with red food color (33,3% (v/v) solution of acid fuchsine available in ROTH®) following the method

described by Thies et al. (2002). Stained roots were washed with tap water and homogenized at 8000 rpm. The number of J2 extracted from the root tissue was counted under a stereomicroscope.

Greenhouse efficacy experiment in eggplant production system

The experiment was conducted in a commercial greenhouse near Svilengrad, Southern Bulgaria. No nematicides had been applied in the greenhouse for at least 2 years prior to the experiment. Plots were 20 m^{-2} (2 m × 10 m) each. The treatments were arranged in randomized complete block design with four replications. One week before planting of the eggplant seedlings all plots were irrigated with 13 L of water m^{-2} and ploughed with rotary cultivator to ensure moisture uniformity. The distances between and within rows were respectively 0.64 and 100 cm. The greenhouse temperature during the cropping period was 18 to 43°C (March-June). Plants were irrigated every three days with a drip system and fertilized every week with compound fertilizer (N: 15 %, P: 15%, K: 15%).

Two weeks eggplant seedlings, cv. Lych were transplanted in the greenhouse on 15 March, 2014. Before planting, the soil into the band had been infested with *M. javanica* at a rate of 5000 eggs and J2 per plant. The experiments included the following treatments: 1. Control with nematodes; 2. Susspension of *P. agglomerans* at dose 10 ml per plant (per treatment) on 1th, 8th and 15th day after transplanting; 3. Susspension of *B. subtilis* at dose 10 ml per plant (per treatment) on 1th, 8th and 15th day after transplanting; 4. Oxamyl (Vydate® 10L) at dose 1 ml plus *P. agglomerans* at dose 10 ml per plant (per treatment) on 8th and 15th day after transplanting; 5. Oxamyl (Vydate® 10L) at dose 1 ml plus *B. subtilis* at dose 10 ml per plant (per treatment) on 8th and 15th after transplanting; 6. Oxamyl (Vydate® 10 L) at dose 1 ml per plant (per treatment) on 1th, 8th and 15th day after transplanting.

The following observations were made: 1. During the cropping season - numbers of females of *M. javanica* (45th day after transplanting the eggplants); 2. At the end of cropping season (94th day after transplanting) - root gall index, numbers of eggs and J2 in the soil (g), and numbers of eggs and J2 in the roots (g). The soil samples on 45th day were taken by means of an auger (2 cm/d) at a distance 10 cm from the plant stem, upon which the roots were separated, washed, stained in acid fuchsine and the numbers of females were counted by direct examination of the roots using a stereomicroscope and determined per gram of fresh roots. At the end of cropping season, the nematodes were extracted from soil samples using a modified Baermann funnel technique (Southey, 1986). Eggs were extracted from roots samples according to hypochlorite procedure (Hussey and Barker, 1973). Root gall index of eggplants was assessed according to a 0 to 10 scale (Bridge and Page, 1980).

Statistical analysis

Obtained data were subjected to one way analysis of variance (ANOVA) and the treatment means were compared with the control plants, according to the Dunnet's test (P<0.05).

RESULTS

Effect of rhizobacteria on the penetration rate of *M. javanica* second stage juveniles

The experiments showed that both tested rhizobacteria influenced the penetration of J2 of *M. javanica* into the eggplant roots (Table 1, P < 0.05). Both, *P. agglomerans* and *B. subtilis* significantly reduced the number of penetrated J2 applied either as a root dipping or soil drench. *B. subtilis* significantly decreased the number of J2 in the roots when was applied as a seed treatment. Reduction of juveniles penetration following seed treatment, root dipping and soil drench ranged to 32.4, 32.0 and 44.6% for *P. agglomerans* and with 38.8, 27.9 and 66.8% for *B. subtilis* compared with the control treatments (Table 1, P < 0.05).

Both, *P. agglomerans* and *B. subtilis* slightly enhanced the eggplants growth. Statistically, differences in both shoot and root weight were not significant neither for *P. agglomerans* nor for *B. subtilis* when applied as a seed treatment, root dipping and soil drench (Table 2, P<0.05). Overall, the eggplants with *P. agglomerans* application showed a better performance in plant growth parameters compared with those treated with *B. subtilis*.

Greenhouse efficacy experiment in eggplant production system

At the mid-season (45th day) plants treated with the rhizobacteria alone or in combination with Oxamyl had significantly lower number of females present in the roots than the plants in the control plots (Table 3, P<0.05). The lowest number of *M. javanica* females was observed in the treatment *B. subtilis* plus Oxamyl (52). *Pantoea agglomerans* plus Oxamyl, as well as Oxamyl applied separately reduced the number of females (78 and 67, respectively) compared with the control (168). Both, *P. agglomerans* and *B. subtilis* affected the development of *M. javanica* J2 and the number of reproductive females was 128 and 111, respectively. At the end of the cropping season, the gall index varied from 5.2 for the plants in the control plots to 1.9 for the plants treated with Oxamyl - *B. subtilis* combination. Significantly fewer galls were also observed on the plant roots in the plots treated with *P. agglomerans* plus Oxamyl (3.0). *Bacillus subtilis*, as well as Oxamyl decreased the number of eggs and J2 in the soil and in the roots, but there was no statistical difference between the treatments. Further, there was no significant difference between *P. agglomerans* and the control in all three parameters of assessment - gall index, eggs and J2 in the soil and eggs and J2 in the plant roots (Table 3, P<0.05).

DISCUSSION

During the last ten years numerous scientific articles have acquainted with achievements of successful *Meloidogyne* control using rhizobacteria (Mendoza et al., 2008; Vetrivelkalai et al., 2010; Majzoob et al., 2012; Bonaterra et al., 2014). In general, our study has also

Table 1. Effect of the rhizobacteria *Bacillus subtilis* and *Pantoea aggromelans* on root penetration of *Meloidogyne javanica* second stage juveniles

Treatments	Seed treatment	Root dipping	Soil drench
P. agglomerans	438.7[a]	426.8[a]	388.1[a]
B. subtilis	397.2[b]	452.5[a]	322.4[a]
Control	649.1[a]	627.6[b]	701.01[b]

Means followed by different letters in the column differ by Dunnet's test (P<0.05).

Table 2. Effect of the rhizobacteria *B. subtilis* and *P. aggromelans* on root and shoot fresh weight of eggplants.

Treatments	Seed treatment		Root dipping		Soil drench	
	Fresh root weight (g)	Fresh shoot weight (g)	Fresh root weight (g)	Fresh shoot weight (g)	Fresh root weight (g)	Fresh shoot weight (g)
P. agglomerans	9.58[a]	13.81[a]	8.72[a]	12.91[a]	8.21[a]	12.17[a]
B. subtilis	8.22[a]	11.82[a]	8.83[a]	12.43[a]	8.04[a]	12.12[a]
Control	8.07[a]	12.01[a]	8.19[a]	12.18[a]	8.02[a]	11.57[a]

Means followed by different letters in the column differ by Dunnet's test (P<0.05).

Table 3. Effect of the rhizobacteria *B. subtilis* and *P. aggromelans* alone and in combination with Oxamyl on numbers of *M. javanica* females, root-gall index, numbers of J2 soil and root in greenhouse experiment.

Treatments	At 45^{-th} day after transplanting	At the end of cropping season		
	Females g^{-1} root	Gall index	Eggs and J2 g^{-1} root	Eggs and J2 g^{-1} soil
Control	168[a]	5.2[a]	9567[a]	256[a]
P. agglomerans	128[b]	4.9[a]	8994[a]	231[a]
B. subtilis	111[b]	4.0[b]	6982[b]	157[a]
Oxamyl + *P. agglomerans*	78[c]	3.0[c]	6548[b]	148[a]
Oxamyl + *B. subtilis*	52[d]	1.9[d]	3947[c]	103[c]
Oxamyl	67[cd]	3.9[b]	6787[b]	171[a]

Means followed by different letters in the column differ by Dunnet's test (P<0.05).

demonstrated the efficacy of the rhizobacteria as biological control agents of *M. javanica* on eggplants. In laboratory pot experiment, *P. agglomerans* and *B. subtilis* caused a reduction of penetration of J2 into the roots up to 44.6 and 66.8%, respectively, following soil drench application. In the same experiment reduction of juvenile penetration was also observed when the bacteria applied as a root dipping. However, the number of J2 in the eggplant roots at this treatment was higher than the number of J2 at the soil drench treatment. Application of *B. subtilis* as a seed treatment resulted in significantly lower number of juveniles in the root systems compared with the control and *P. agglomerans* treatment. Although, the fresh root and shoot weight of eggplants treated with *P. agglomerans* and B. subtilis were higher compared with the control the differences were not significant. Amellal et al. (1999) reported that *P. agglomerans* is a competent colonizers of the rhizosphere and therefore a promising candidate for biological control. Plant-beneficial effect of *P. agglomerans* was observed by Majzoob et al. (2012) and Bonaterra et al. (2014) on cucumber and tomato, both infested with *M. javanica*. Several *Bacillus* strains have been assessed for their potential as a biological control agent of *Meloidogyne* species. Mendoza et al. (2008) reported *in vitro* activity of a *Bacillus firmus* strain against eggs and J2 of *M. incognita*. Strains of *Bacillus megaterium*, *Bacillis pumilus* and *Bacillus mycoides* reduced the number of egg masses of *M. incognita* on tomato by 31, 30% and 39% respectively (Mekete et al., 2009). The results obtained in the current experiments indicate that the effects of rhizobacteria treatments were similar to those described above. Our results also support the finding of Munif et al. (2013), who reported that *P. agglomerans* reduced *M. incognita* J2 root penetration by 31% following soil drench application. In addition, the authors discussed that application of endophytic bacteria with root dipping and soil drench is more effective for suppressing the penetration of nematode juveniles compared with seed treatment application of (Munif et al., 2013).

According to Thies et al. (1992) biological control of root-knot nematodes usually has not been a stand-alone practice in control of these nematodes. Our results on the other hand, showed that *B. subtilis* can potentially reduce *M. javanica* population in a greenhouse up to the end of the cropping season. The suppressive effect was observed within 45 days after transplanting eggplants and continued through the end of the greenhouse experiment. In addition, the obtained data from this experiment indicate that the chemical nematicides such as carbamates are more effective at the beginning of cropping season, and their efficiency is higher in combinations with the tested bacteria. Furthermore, *P. agglomerans* applied separately does not provide long-lasting protection of plants. However, in the plots treated with *P. agglomerans* plus Oxamyl, final *M. javanica* population was significantly lower than in the control plots. Suppression of the *M. javanica* populations throughout the plant growth is probably due to the successful colonization of the plant rhizosphere and good multiplication rates of the *B. subtilis* and *P. agglomerans*. This is consistent with previous findings reporting successful control of root-knot nematodes using bacteria under greenhouse condition (Giannakou et al., 2004). These authors reported that *B. firmus* significantly reduced the root-knot index on cucumber plants in a greenhouse in Greece. Similarly, Majzoob et al. (2012) stated suppression of *M. javanica* reproduction levels on greenhouse cucumbers in Iran.

It is important to note that although the rhizobacteria reduced the gall index the effect was not uniform, and eggplants within individual plots showed certain variation in the gall index. Moreover, the heavily galled plants suffered from secondary infection by soil-borne fungi (data not presented). This might be the reason for a few dead plants observed at the end of cropping season. Hence, the application of bacteria and chemicals, whose efficacy is limited to nematodes, should be accompanied by additions of fungicides to prevent infestation by soil-borne fungi, such as *Fusarium* sp. and *Verticillium* sp.

Conclusion

The rhizobacterium *B. subtilis* showed promising results in reduction of *M. javanica* reproduction in eggplant and could be consider as a good non-chemical alternative for the control of root-knot nematodes. The superior performance of the *B. subtilis* in combination with an organophosphate nematicide (Oxamyl) in comparison with their separate application, suggests that this bacteria may be successfully included in an integrated scheme for the control of root-knot nematodes.

Conflict of Interests

The authors have not declared any conflict of interests.

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