

THE INFLUENCE OF ENDOPHYTIC BACTERIA ON *Meloidogyne incognita* INFECTION AND TOMATO PLANT GROWTH

Abdul Munif¹, Johannes Hallmann², Richard A Sikora²

¹Department of Plant Protection, Bogor Agricultural University, Indonesia
Jl. Kamper Kampus IPB Darmaga, Bogor, Indonesia

²Institut fuer Pflanzenkrankheiten, University of Bonn, Germany
Nussallee 9 D-53119 Bonn, Germany

Corresponding author: abdulmunif@ipb.ac.id

(Received: April 19, 2013; Accepted: November 30, 2013)

ABSTRACT

Plant parasitic nematodes cause significant damage and losses to vegetable crops. Control of plant parasitic nematodes with pesticides is often restricted due to their high toxicity and negative impact on the environment. The need for environmentally safe control strategies has increased interest in developing biological control measures. The objective of this research was to study the effect of selected endophytic bacteria, *Pantoea agglomerans* MK-29, *Cedecea davisae* MK-30, *Enterobacter* spp. MK-42 and *Pseudomonas putida* MT-19 on early root penetration and gall formation of *Meloidogyne incognita* on tomato. The results showed that application of bacterial endophytes significantly reduced *M. incognita* infestation on tomato either as a seed treatment, root dipping or as a soil drench application. The four selected endophytic bacteria also significantly reduced early root penetration of *Meloidogyne* juveniles into tomato roots up to 56 %, when applied as a root dipping and soil drench. Seed treatment of endophytic bacteria followed by soil drench application apparently gave a higher reduction in the number of galls than the single application.

Key words: Gall, seed treatment, root dipping, soil drench.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are an important group of plant parasitic nematodes that have a world-wide distribution, extensive host ranges and are able to interact with other plant-parasitic nematodes and other pathogens to form complex disease syndromes (Agrios, 2005). Species of *Meloidogyne* cause severe damage to many crop plants, especially vegetable crops. Crop losses due to *Meloidogyne* exceed 32% on tomato, 30% on melon and 20% on eggplant (Netscher and Sikora, 1990). More than 50 species of *Meloidogyne* are known to occur world-wide, but four species are of particular importance to vegetable production, *M. incognita*, *M. javanica*, *M. arenaria* and *M. Hapla* (Sasser, 1979).

Plant parasitic nematodes living belowground are difficult to control by chemical means because of large quantities and repeated applications required to treat the entire soil volume occupied by plant roots. A number of antagonistic bacteria have been reported in suppressing soil-borne pathogens and enhancing plant growth. An advantage of targeted introduction of antagonists to the plant is that microbial populations can grow from a small quantity of inoculum and colonize the rhizosphere and root (Sikora, 1992). Endophytic bacteria are bacteria that live inside plant tissues without doing symptoms on these plants. As the internal plant habitat, endophytic bacteria provide several advantages as biological control agents, namely the colonization of an ecological niche also used by plant pathogens, less competition with other microorganisms, sufficient supply with nutrients,

less exposure to environmental stress factors, and better translocation of bacterial metabolites throughout the host plant (Hallmann et al., 1997).

The most extensively studied endophytic bacteria include *Pseudomonas* spp. *Bacillus* spp. *Serratia* spp. and *Enterobacter* spp. which have been reported as a biocontrol agents to reduce fungal diseases (Yang et al., 2011), bacterial diseases (Sarr et al., 2010) and plant parasitic nematodes (Munif et al., 2000; Vetrivelkai et al., 2010). The low level of control consistency of many biocontrol agents against soil-borne pathogens under field conditions is most likely due to the complexity and variability of the soil physics, chemistry and microbial activity in the soil as well as due to environmental factors (Weller, 1988). Therefore, achieving efficient and consistent performance of biocontrol agents requires a more thorough knowledge of effective screening techniques, regulation of biotic and abiotic factors and the development of acceptable formulation and application techniques. The objective of the present studies were to study the effect of endophytic bacteria on early root penetration and gall formation of *M. incognita* and their effect on the plant growth.

MATERIALS AND METHODS

Strains of endophytic bacteria

Endophytic bacteria was isolated from tomato roots. The isolation procedure was done using surface sterilization method with alcohol 70% and sodium hypochlorite 3% (Hallmann et al., 1997). Four strains of the selected endophytic bacteria, *Pantoea agglomerans* MK-29, *Cedecea davisae* MK-30, *Pseudomonas putida* MT-19, and *Enterobacter* spp. MK-42 presvously reported significantly reduce galls of nematodes incidence on tomato were used in this study.

Effect of endophytic bacteria on the penetration rate of *Meloidogyne* juveniles

The effect of endophytic bacteria on juvenile penetration was tested using 3 application methods, i.e. seed treatment, root dipping and soil drench. The strains of endophytic bacteria tested were pre-cultured on tryptic soy agar (TSA) for 48 hours at 24°C. A loop of the bacteria was then transferred into 100 ml tryptic soy broth (TSB) and incubated on a shaker at 100 rpm and 22-24°C for 48 hours. The bacterial suspension was centrifuged at 4°C for 20 min at 4,600 x g (Haereus Varifuge RF), and the bacterial cells in the pellet were resuspended in sterile 1/4 strength Ringer's solution (Merck). The bacterial suspension was adjusted to $OD_{560}=2.0$ by dilution with Ringer's solution. The bacteria were applied as follows:

Seed treatment. The bacteria were pre-cultured on TSA and then resuspended in 4 ml of 2% methyl cellulose solution. The tomato seeds c.v. Hellfrucht Frühstamm were soaked in the bacterial suspension for 30 minutes and then seeded into pots containing an unsterilized soil/sand mixture (1:1,v/v). Each pot received 3 seeds. After 2 weeks, plants were thinned to one plant per pot. After 4 weeks, the plants were inoculated with 2000 juveniles of *M. incognita* per pot. The inoculation of nematodes was carried out by drenching 3 ml inoculum volum with the juveniles into the potted soil around the roots. Each treatment was replicated 6 times. The experiment was terminated 6 weeks after nematode inoculation. Tomato plant roots were washed free of adhering soil particles using tap water and the shoot and root fresh weights as well as the number of galls were recorded.

Root dipping. Roots of three-week-old tomato plants were dipped for 30 min into the bacterial suspension and then planted into pots containing an unsterilized soil/sand mixture (1:1,v/v). After 2 weeks, the plants were inoculated with 2000 juveniles of *M. incognita* per pot. Each treatment was replicated 6 times and terminated 6 weeks after nematode inoculation.

Soil drench: Three ml bacterial suspensions were pipetted onto the soil surface around 3-week-old tomato plants. Plants were inoculated with 2000 juveniles of *M. incognita* 6 days after bacterial application. The inoculation of nematodes was carried out by drenching 3 ml inoculum volume with the juveniles into the potted soil around the roots. Plants were harvested after 6 weeks. Fresh shoot and root weights were measured and the penetration rate of *M. incognita* was recorded. The penetration rate of *Meloidogyne* juveniles into tomato roots was determined after staining the roots with 0.1 % lactic acid fuchsin (789 ml lactic acid, 56 ml glycerol, 1 g acid fuchsin, and 154 ml distilled water). The tomato roots were transferred into 100 ml glass tubes, 50 ml of 0.1% lactic acid fuchsin was added and the tubes were heated to boiling in the microwave for 30 seconds. They were then stored at room temperature for at least 24 hours (Hussey and Barker, 1973). To determine the juvenile penetration rate, stained roots were washed with tap water and homogenized in an Ultra-Turrax (IKA-Werk) at 10,000 rpm. The number of juveniles released from the root tissue was counted under a stereomicroscope.

Effect of seed treatment followed with soil drench of endophytic bacteria

The tomato seeds c.v. Hellfrucht Frühstamm were soaked in the bacterial suspension for 30 minutes and then seeded into pots containing an unsterilized soil/sand mixture (1:1,v/v). Each pot received 3 seeds. After 2 weeks, plants were thinned to one plant per pot. Two weeks later, 5 ml of a bacterial suspension ($OD_{560}=2.0$) was applied onto each plant as a soil drench near the plants. After another 5 days, the plants were inoculated with 2000 juveniles of *M. incognita* per pot. The inoculation of nematodes was carried out by drenching 3 ml inoculum volume with the juveniles into the potted soil around the roots. Each treatment was replicated 6 times. The experiment was terminated 6 weeks after nematode inoculation. Tomato plant roots were washed free of adhering soil particles using tap water and the shoot and root fresh weights as well as the number of galls were recorded.

RESULTS AND DISCUSSION

Effect of endophytic bacteria on the penetration of *Meloidogyne*

The biocontrol potential of endophytic bacteria to plant parasitic nematodes is well reported (Hallmann et al., 1997; Munif et al., 2000; Vetrivelkai et al., 2010). Little is known about the mechanism of endophytic bacteria for controlling nematodes. Endophytic bacteria colonize a similar niche like many endo-parasitic nematodes and therefore are good biocontrol candidates. Especially, sedentary plant parasitic nematodes are primary target organisms for biological control with endophytic bacteria, because they spend most of their life cycle in the root (Sikora, 1992).

These experiments showed that endophytic bacteria influenced the penetration and development of *M. incognita* in the plant roots. The bacteria were applied either as a seed treatment, root dipping or soil drench. All 4 endophytic bacteria significantly reduced penetration of *Meloidogyne* juveniles into tomato roots, when applied as a soil drench. Following root dipping, *C. davisae* MK-30 and *Enterobacter* spp. MK-42 significantly reduced penetration, whereas seed treatment showed no effect in this experiment. Reductions in *M. incognita* penetration following soil drench application ranged from 31% for *P. agglomerans* to 56% for *Enterobacter* spp. MK-42 and with root dipping from 21% to 45% for *P. putida* MT-19 and *C. davisae* MK-30, respectively. 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. Supposedly, soil drench or root dipping provided higher inoculum densities of endophytic bacteria (Table 1).

The most effective bacteria from the screening process showed strong antagonism towards *M. incognita* in all tests. Especially *P. agglomerans* was effective, a well known antagonist reported

to suppress fire-blight of pear and apple caused by *Erwinia amylovora* (Johnson et al., 2000). Mechanisms suggested to be involved in pathogen suppression include competition for iron and space, and induced systemic resistance (Braun et al., 1998). Amellal et al. (1999) reported that *P. agglomerans* is a competent colonizers of the rhizoplane and rhizosphere and therefore a promising candidate for biological control.

Specific *Pseudomonas sp.* were reported to suppress several plant parasitic nematodes, namely *Globodera pallida* (Racke and Sikora, 1992), *Heterodera schachtii* (Neipp and Becker, 1999), *Meloidogyne incognita* (Becker et al., 1988; Hallmann et al., 1998), *Meloidogyne javanica* (Spiegel et al., 1991) and *Pratylenchus penetrans* (Hackenberg et al., 2000). *Pseudomonas* control mechanisms include competition, metabolites with nematicidal activity and induced resistance. However, the exact mode of action of most endophytic *Pseudomonas spp.* for the biological control of plant parasitic nematodes requires further investigation.

Table 1. Effect of the endophytic bacteria on early root penetration of *Meloidogyne incognita* juveniles following seed treatment, root dipping or soil drenching.

Treatments	Seed treatment	Root dipping	Soil drench
Control	142 abc	370 a	142 a
<i>P. agglomerans</i> MK-29	189 a	263 ab	98 b
<i>C. davisae</i> MK-30	101 c	203 b	83 bc
<i>Enterobacter spp.</i> MK-42	189 a	234 b	63 c
<i>P. putida</i> MT-19	177 ab	291 ab	86 bc

Means followed by the same letter within a column are not significantly different according to Duncan's Multiple Range Test ($P=0.05$, $n=6$)

Endophytic bacteria on the plant growth

All endophytic bacteria apparently increased fresh shoot weights and root lengths of the tomato plants. The highest root length and fresh shoot weight was reached by strain MK-29 when the endophyte applied as a seed treatment, whereas the highest root length and fresh shoot weight was reached by strain MK-30 when applied as a root dipping or soil drench. Statistically, differences in total root lengths were significant for strains *C. davisae* MK-30, *Enterobacter spp.* MK-42 and *P. putida* MT-19 when applied as a soil drench (Table 2).

Table 2. Effect of the endophytic bacteria *P. agglomerans* MK-29, *C. davisae* MK-30, *Enterobacter spp.* MK-42 and *P. putida* MT-19 applied as a seed treatment, root dipping or soil drench on root length and shoot fresh weight of tomato plants

Treatments	Seed treatment		Root dipping		Soil drench	
	Total root length (cm)	Fresh shoot weight (g)	Total root length (cm)	Fresh shoot weight (g)	Total root length (cm)	Fresh shoot weight (g)
Control	11.02 a	3.06 a	7.58 a	3.60 a	9.86 b	3.67 a
MK-29	11.45 a	3.32 a	7.41 a	3.62 a	9.01 b	4.61 a
MK-30	10.35 a	3.16 a	8.12 a	4.39 a	11.56 a	4.99 a
MK-42	11.22 a	3.08 a	6.52 a	3.38 a	11.13 a	4.81 a
MT-19	11.34 a	3.28 a	7.60 a	3.99 a	11.42 a	4.32 a

Means followed by the same letter within a column are not significantly different according to Duncan's Multiple Range Test ($P=0.05$, $n=6$).

Effect of seed treatment combined with soil drench of endophytic bacteria

P. agglomerans MK-29, *C. davisae* MK-30, *E. intermedius* MK-42 and *P. putida* MT-19 reduced the number of root galls and increased fresh shoot weights of tomato plants when applied as a seed treatment combined with soil drench (Table 3). The differences in root galls were significant for the strains MK-29, MK-30 and MT-19. Reductions in root galls were 23% and 29% compared to the non-treated plants. Bacterial application caused an increase in fresh shoot weight up to 21% and fresh root weight up to 25% (Table 3). Differences in fresh shoot weight were significant for strain MK-29, MK-30 and MT-19 and fresh root weight for strain MK-29 and MT-19.

Table 3. The effect of seed treatment followed by a soil drench application of the endophytic bacteria, on the number of root galls, fresh shoot weights and fresh root weights of tomato infected with *M. incognita*.

Treatments	Number of root galls	Fresh root weight (g)	Fresh shoot weight (g)
Control	125 a	2.78 c	8.84 b
<i>P. agglomerans</i> MK-29,	94 b	3.36 ab	10.82 a
<i>C. davisae</i> MK-30	90 b	2.99 abc	10.91 a
<i>E. intermedius</i> MK-42	107 ab	2.85 bc	9.98 ab
<i>P. putida</i> MT-19	98 b	3.40 a	11.09 a

The present experiment showed that seed treatment of endophytic bacteria followed by a soil drench with a single endophytic bacteria increased nematode control and suppressed the number of galls of nematode as shown for *P. agglomerans* MK-29, *C. davisae* MK-30, *Enterobacter* spp. MK-42, and *P. putida* MT-19. Dual application of endophytic bacteria was assumed to increase the density of the bacterial antagonist in the soil. With seed treatment, the antagonist is placed on the seed surface and may protect the seedling from germination onward. However, bacterial growth along the developing root is often limited. A soil drench application 14 days later may deliver additional bacteria onto newly grown root tips and increase antagonistic activity. Racke and Sikora (1992) reported that *A. radiobacter* and *B. sphaericus* at population densities of 9.7×10^8 and 3.16×10^9 cfu/ml respectively, were required for significant reductions in root penetration by *Globodera pallida* on potato. Reduced nematode penetration was more pronounced following a soil drench and root dip application than for seed treatment. Reduction in nematode penetration is most likely caused by alterations of root exudate patterns (Sikora, 1992). Root exudates affect hatching, attraction, repellence and invasion of nematodes (Perry and Gaur, 1996).

The complexity of the soil ecosystem makes biological control of soil borne pathogens or plant parasitic nematodes a challenge (Backman and Sikora, 2008). The establishment of high densities of an antagonist is a key factor in biological control. The future use of the unique endophytes could be to directly treat seeds or transplants or root dipping; limiting substantially the side-effects of abiotic and biotic factors on the biological agent by almost immediately protecting them within plant tissues.

CONCLUSION

The selected bacterial endophytes isolated from tomato, *P. agglomerans* MK-29, *C. davisae* MK-30, *E. intermedius* MK-42 and *P. putida* are able to reduce the number of penetrating nematodes and root galls of tomato when applied as a root dipping and soil drench. Seed treatment of endophytic

bacteria followed by a soil drench with single endophytic bacteria increased nematode control and suppressed the number of galls nematode. Endophytic bacteria provide close interaction with plant tissue and make them ideal candidates for the biological control of plant parasitic nematodes.

ACKNOWLEDGEMENT

This research was financially supported by German Student Exchange (DAAD).

REFERENCES

- Agrios, G.N. 2005. Plant Pathology, 5th Edition. Academic Press. New York.
- Amellal, A., Bartoli F., Villamin G., Talouizte A. and T. Heulin. 1999. Effects of inoculation of EPS-producing *Pantoea agglomerans* on wheat rhizosphere aggregation. Plant and Soil 211:93-101.
- Backman, P.A. and R.A. Sikora. 2008. Endophytes: An emerging tool for biological control. Biol. Control 46 (2008) 1–3. doi:10.1016/j.biocontrol.2008.03.009.
- Becker, J.O., Zavaleta-Meija E., Colbert S.F., Schroth M.N., Weinhold A.R., Hancock J.G. and S.D. Van Gundy. 1988. Effect of rhizobacteria on root-knot nematodes and gall formation. Phytopathology 78:1466-1469.
- Braun, A., Sands D.C. and B.J. Jacobsen. 1998. Mechanism in the biological control of *Pseudomonas syringe* pv. *Syringe* by *Pantoea agglomerans*. IOBC Bull. 21(9):7-12.
- Hussey, R.S. and K.R. Barker. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including new technique. Plant Disease Rep. 57(12):1025-1028.
- Hackenbarg. C., Muehlchen A., Forge T. and T. Vrain. 2000. *Pseudomonas chlororaphis* strain Sm3, bacterial antagonist of *Pratylenchus penetrans*. J. Nematol. 32(2):183-189.
- Hallmann, J., Quadt-Hallmann A., Mahaffee W.F. and J.W. Kloepper. 1997. Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43:895-914.
- Hallmann, J., Quadt-Hallmann A., Rodriguez-Kabana R. and J.W. Kloepper. 1998. Interaction between *Meloidogyne incognita* and endophytic bacteria in cotton in relation to nematode control. Soil Biol. Biochem. 30 (7):925-937.
- Johnson, K.B., Stockwell V.O., Sawyer, T.L. and D. Sugar. 2000. Assessment of environmental factors influencing growth and spread of *Pantoea agglomerans* on and among blossoms of pear and apple. Phytopathology 90:1285-1294.
- Munif, A., Hallmann J. and R.A. Sikora. 2000. Evaluation of the biocontrol activity of endophytic bacteria from tomato against *Meloidogyne incognita*. Med. Fac. Landbouww. Univ. Gent 65:471-480.
- Neipp, P.W. and J.O. Backer. 1998. Evaluation of biocontrol activity of rhizobacteria from *Beta vulgaris* against *Heterodera schachtii*. J. Nematol. 31(1):54-61.
- Netscher, C. and R.A. Sikora. 1990. Nematode parasite on vegetables. In: Luc M, Bridge J and Sikora RA (Eds.). Plant Parasitic Nematode in Subtropical and Tropical Agriculture. CAB International, Wallingford, UK.pp 237-284.

- Perry, R.N. and H.S. Gaur. 1996. Host plant influences on the hatching of cyst nematodes. *Fund. Appl. Nematol.* 19:505-510.
- Racke, J. and R.A. Sikora. 1992. Isolation, formulation and antagonistic activity of rhizobacteria toward the potato cyst nematode *Globodera pallida*. *Soil Biol. Biochem.* 24(6):521-526.
- Sasser, J.N. 1979. Pathogenicity, host range and variability in *Meloidogyne* spp. In: F. Lamberti and C.E. Taylor (Eds.). *Root-Knot Nematode (Meloidogyne spp.): Systematic, Biology and Control*. Academic Press. London. p. 257-268.
- Sarr, P.S., Yamakawa T., Asatsuma S., Fujimoto S. and M. Sakai. 2010. Investigation of endophytic and symbiotic features of *Rashtonia* sp. TSC1 isolated from cowpea nodules. *Afr. J. Microbiol. Res.* 4:1959-1963.
- Sikora, R.A. 1992. Management of the antagonistic potential in agricultural ecosystem for the biological control of plant parasitic nematodes. *Annu. Rev. Phytopathol.* 30:245-270.
- Spiegel, Y., Cohn E., Galper S., Sharon E. and I. Chet. 1991. Evaluation of a newly isolated bacterium, *Pseudomonas chitinolytica* for controlling the root-knot nematode *Meloidogyne javanica*. *Biocontrol Sci. Tech.* 1:115-125.
- Vetrivelkai, P., Sivakumar M. and F.I. Jonathan. 2010. Biocontrol potential of endophytic bacteria on *Meloidogyne incognita* and its effect on plant growth in bhendi. *J. Biopest.* 3:52-457.
- Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* 26:379-407.
- Yang, C.J., Zhang X.G., Shi G.Y., Zhao H.Y., Chen L., Tao K. and T.P. Hou. 2011. Isolation and Identification of endophytic bacterium W4 against tomato *Botrytis cinerea* and antagonistic activity stability. *Afr. J. Microbiol. Res.* 5:131-136.