EVALUATION OF THE BIOCONTROL ACTIVITY OF ENDOPHYTIC BACTERIA FROM TOMATO AGAINST *MELOIDOGYNE INCOGNITA*

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SUMMARY

Endophytic bacteria are ubiquitous in most plant species and reside within healthy plant tissue without producing symptoms of damage. Some endophytic bacteria have shown potential to improve plant growth and reduce disease symptoms caused by several plant pathogens. Little information is available regarding the effect of endophytic bacteria on plant parasitic nematodes. The objectives of this study were to evaluate and characterize the biocontrol activity of endophytic bacteria isolated from and applied to tomato on gall formation caused by *Meloidogyne incognita* and to study the internal colonization of two selected bacterial isolates. Fifteen out of 120 endophytic bacterial isolates were repeatedly tested for their biocontrol and plant growth promoting potential using either soil drench or seed application. Four out of these 15 isolates significantly reduced numbers of *M. incognita* galls following soil drench application and 6 isolates significantly reduced nematode infestation when applied as a seed treatment. In addition, several endophytic bacteria improved plant growth significantly. Colonization of tomato roots by endophytic bacteria was found to be stable for at least six weeks. The results demonstrated that endophytic bacteria consistently colonize the internal plant tissue of their host and have biocontrol and plant growth promoting potential.

INTRODUCTION

Control of plant parasitic nematodes with nematicides 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 (SIKORA, 1992), whereas biological control within this context will be defined as the use of antagonistic organisms to control pathogen populations. In general, microorganisms in the rhizosphere provide a first defense line to protect the root from pathogen attack (WELLER, 1988). The presence of rhizobacteria can significantly modify the rhizosphere environment and affect directly or indirectly soil-borne diseases and pests (COOK & BAKER, 1983; SIKORA, 1992). More recently, awareness of the presence of tissue colonization with rhizobacteria marked the beginning of a new research area, i.e. the importance of endophytic bacteria. For use as plant protection agents, the internal habitat provides several advantages for the bacteria when compared with the rhizosphere: 1) colonization of an ecological niche also used by plant pathogens, 2) less competition with other microorganisms, 3) sufficient supply of nutrients, 4) less exposure to environmental stress factors, and 5) better translocation of bacterial

metabolites throughout the host plant (summarized in HALLMANN et. al., 1997). Due to their close association with the plant and pathogens, endophytic bacteria are considered to be ideal biocontrol candidates. The objectives of this work were to 1) evaluate the effect of endophytic bacteria isolated from tomato roots on *M. incognita* infestation of tomato using soil drench and seed application, and 2) to study the internal population dynamics of two selected endophytic bacteria over time inside the root tissue.

MATERIALS AND METHODS

Isolation, culture, and identification of bacteria. The bacteria were originally isolated from tomato roots grown in Germany and Indonesia. Roots were washed and surface sterilized in 3% NaOCl (a.i.) and 0.01% Tween 20 for 3 minutes. The roots were then washed three times in 0.1 M sterile potassium phosphate buffer (PB) and homogenized with mortar and pestle in PB under aseptic conditions. Following serial dilution the bacterial suspension was plated on 1/10 tryptic soy agar (TSA). Following incubation at 28°C for 48 hours single bacterial colonies were randomly selected and purified on full strength TSA. The bacterial isolates were finally stored in tryptic soy broth (TSB) plus 20% glycerol at –80°C. The bacteria were identified using fatty acid analyses (FAME) and MIDI system. The bacterial identification of the 15 isolates used within this studies are given in Table 1.

Table1. Identification of selected endophytic bacteria isolated from tomato internal tissue using FAME-GC

Strains	Bacterial species	Similarity index [a)]
MT-04	*Pseudomonas putida*	0.080
MT-09	*Pseudomonas chlororaphis*	0.858
MT-17	*Kluyvera cryocrescens*	0.801
MT-19	*Pseudomonas putida*	0.940
MK-12	*Cellulomonas flavigena*	0.720
MK-29	*Pantoea agglomerans*	0.673
MK-30	*Cedecea davisae*	0.551
MK-34	*Bacillus megaterium*	0.696
MK-35	*Pseudomonas fluorescens*	0.326
MK-42	*Enterobacter intermedius*	0.317
MK-43	*Cedecea davisae*	0.656
MK-45	*Pseudomonas savastanoi* pv. *fraxinus*	0.904
MK-54	*Pseudomonas stutzeri*	0.302
MK-62	*Pseudomonas mendocina*	0.162
MK-66	*Pantoea agglomerans*	0.886

[a)] Similarity index (SI) of the 15 bacterial strains used within these studies based on their fatty acid profiles. Identification at the genus or species level was considered good at SI >0.2 or >0.4, respectively, and when the difference from the next respective match was greater than 0.1.

Soil drench application. The bacterial strains were pre-cultured on TSA for 2 days at 28°C. A loop of bacteria was transferred into liquid medium TSB and aggetated for two days at 24°C. The liquid culture was centrifuged at 7500 rpm for 20 minutes. The bacterial pellet was resuspended in ¼ strength Ringer's solution (Merck KGaA, Darmstadt, Germany) and adjusted photometrically to OD_{560}=2.0, representing 10^9-10^{10} cfu/ml depending on the bacterial strain used. Five ml of the bacterial suspension was inoculated onto each plant per pot as a soil drench. Control plants received 5 ml Ringer's solution. Six days later, 700 juveniles of *M. incognita* were inoculated into the root zone of each plant. Five weeks after nematode inoculation, the plants were harvested and the shoot fresh weight, root length as well as the number of galls were recorded. The root length was measured using a standard scanner driven by the software WinRhizo (Regent Instruments Inc., Quebec, Canada). The tomato cultivar "Hellfrucht Frühstamm", which is highly susceptible to *M. incognita*, was used in all experiments. The tomatoes were planted in pots containing 500 cm^3 soil/sand mixture (1:2, v:v) and fertilized biweekly with 0.2 % of a standard fertilizer solution. The experiment was organized in a completely randomized block design with 10 replicates per treatment.

Seed treatment. Bacterial strains were cultured on TSA for 2 days at 28°C. The bacteria were scraped and resuspended in 5 ml of a 1% methyl cellulose solution. Tomato seeds were incubated in the bacterial suspension for 30 minutes, and finally dried on sterile filter paper under the laminar flow hood for 2 hours. The dried seeds were planted in pots with 500 cm^3 soil/sand mixture (1:2, v:v). Two weeks later, 700 juveniles of *M. incognita* were inoculated per plant and after another 5 weeks the experiment was terminated. Shoot fresh weight, root length and number of nematode galls were recorded as described above. The experiment was set up as a completely randomized block design with 10 replicates per treatment.

Internal root colonization. The 2 endophytic bacterial isolates *Pantoea agglomerans* MK-29 and *Pseudomonas putida* MT-19 were used to study the internal root colonization of endophytic bacteria on tomato. Spontaneous rifampicin-resistant mutants were produced by growing the bacteria on TSA containing 50 ppm rifampicin. Mutants selected under these condition were then cultured on TSA amended with 100 ppm rifampicin. Growth of bacterial mutants was then compared to that of the wild type on antibiotic-free media. Tomato was sown into pots containing 500 cm^3 soil/sand mixture (1:2, v:v). Three days later, 10 ml of a bacterial suspension was added to the root zone of each plant as a soil drench. The control treatment was inoculated with sterile water. The experiment consisted of a completely randomized design with eight replicates. At one-week intervals beginning one week after inoculation, a sub-sample of 3

plants was removed from the experiment and the roots were gently washed with tap water and weighed. The roots were surface sterilized with 1.5 % NaOCl (a.i.) for 3 minutes, washed with sterile water 4 times and then ground in PB using a mortar and pestle. Serial dilutions were prepared and 0.1 ml aliquots of the bacterial suspension were streaked on 1/10 strength TSA containing 100 ppm rifampicin. Plates were incubated at 24°-26°C for 48 hours before colonies were counted.

RESULTS

Plant growth. Several of the endophytic bacteria tested were able to improve plant growth significantly (Table 2). Four endophytic bacterial isolates significantly increased the plant's shoot fresh weight following both application techniques as a soil drench or as seed application (*Pantoea agglomerans* MK-29, *Cedecea davisae* MK-30, *Pseudomonas savastanoi* pv. *fraxinus* MK-45 and *Pantoea agglomerans* MK-66). An additional 2 isolates, MT-04 and MK-42, increased shoot fresh weight when applied to the seed. The highest shoot fresh weight was achieved by MK-30. Shoot growth stimulation compared with the control was 30% for soil drench and 24% for seed application.

Table 2. Effect of selected endophytic bacteria on shoot fresh weight and total root length of tomato plants infested with *Meloidogyne incognita* using two application methods

Strains	Soil drench		Seed treatment	
	Shoot weight (g)	Total root length (m)	Shoot weight (g)	Total root length (m)
Control	5.71 de	16.98 ab	5.85 efg	16.43 ab
M.incognita	5.62 e	14.38 e	5.20 g	13,93 ef
MT-04 + *M.i.*	6.48 bcde	16.85 abc	6.65 bcd	16.47 ab
MT-09 + *M.i.*	6.43 bcde	16.28 abcd	6.27 cde	15.93 ab
MT-17 + *M.i.*	6.05 cde	14.68 de	6.00 cde	14.25 cdef
MT-19 + *M.i.*	6.52 bcde	16.75 abc	6.25 cde	16.30 ab
MK-12 + *M.i.*	6.23 bcde	16.20 abcd	5.83 efg	15.65 bc
MK-29 + *M.i.*	6.83 bc	16.57 abc	6.63 bcd	16.18 ab
MK-30 + *M.i.*	7.97 a	15.93 bcde	7.70 a	15.45 bcd
MK-34 + *M.i.*	6.58 bcd	15.63 bcde	6.43 bcde	15.15 bcdef
MK-35 + *M.i.*	5.70 de	14.27 e	5.47 fg	13.67 f
MK-42 + *M.i.*	6.62 bcd	16.13 abcd	6.88 bc	15.93 ab
MK-43 + *M.i.*	6.10 cde	15.67 bcde	5.78 efg	15.40 bcde
MK-45 + *M.i.*	7.10 ab	15.22 cde	6.97 abc	15.20 bcde
MK-54 + *M.i.*	5.98 cde	14.27 e	6.03 def	14.10 def
MK-62 + *M.i.*	6.17 cde	15.58 bcde	5.98 def	15.12 bcdef-
MK-66 + *M.i.*	6.65 bc	17.70 a	7,13 ab	17.25 a

Data followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test.

The highest root length was achieved by MK-66 for both soil drench (17.7 m) and seed application (17.25 m), whereas the lowest root length was measured for MK-35 with 14.27 m and 13.67 m, respectively (Table 2). The root length of nematode infested plant was generally lower than in the non infested control plants. Important was the fact that root length was significantly increased by 7 bacterial isolates applied as a soil drench and 9 isolates applied as a seed treatment.

Nematode infestation. Following the soil drench application, four isolates *Pantoe agglomerans* MK-29, *Cedecea davisae* MK-30, *Enterobacter intermedius* MK-42 and *Pseudomonas putida* MT-19 significantly reduced the number of galls compared with the control (figure 1). When seed application was used, the 6 isolates *Pseudomonas putida* MT-04, *Pantoea agglomerans* MK-29, *Cedecea davisae* MK-30, *Pseudomonas putida* MT-19, *Enterobacter intermedius* MK-42 and *Pseudomonas fluorescens* MK-35 gave significant nematode control in terms of reduced galling (figure 2). The 4 isolates MK-29, MK-30, MT-19 and MK-42 significantly reduced the number of galls in both application techniques. The reduction in number of galls of these 4 isolates ranged between 27% to 43%.

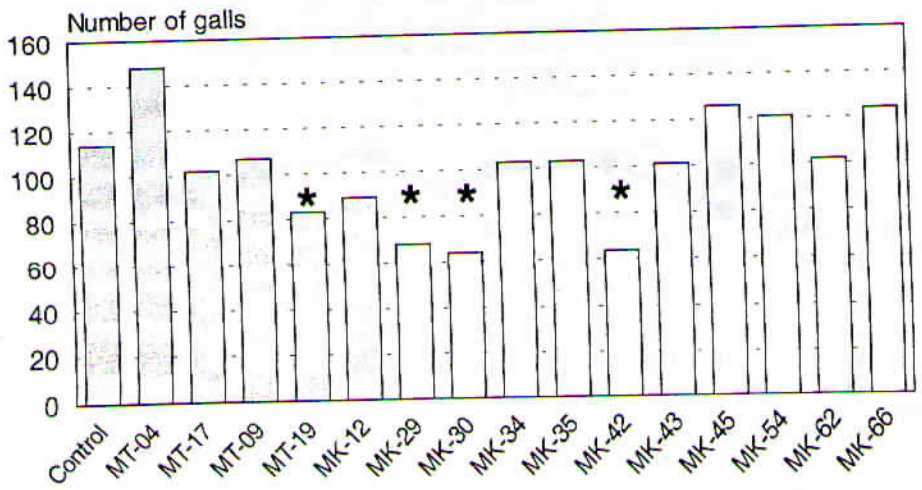

Figure 1. Effect of selected endophytic bacteria applied as a soil drench on the number of galls caused by *Meloidogyne incognita* on tomato. The asterix indicates those bacterial isolates which significantly reduced the number of galls compared with the nematode infested control ($P = 0.05$, n = 10).

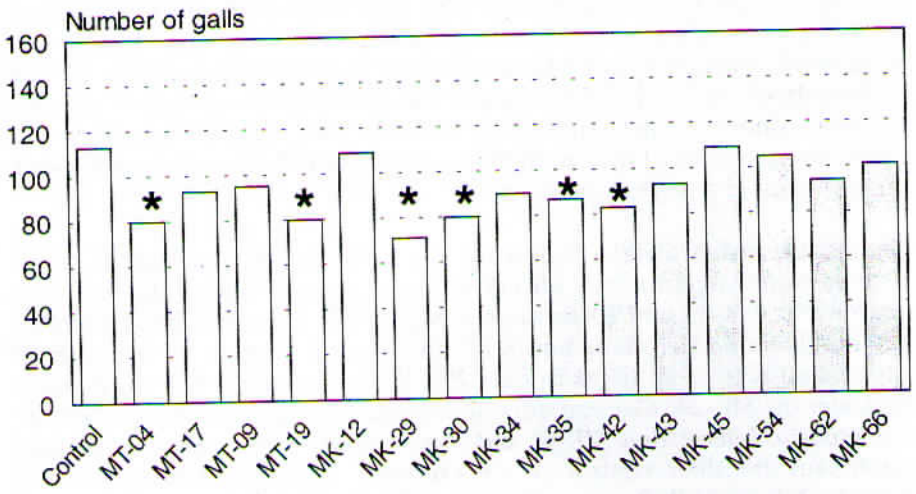

Figure 2. Effect of selected endophytic bacteria applied as a seed treatment on the number of galls caused by *Meloidogye incognita* on tomato. The asterix indicates those bacterial isolates which significantly reduced the number of galls compared with the nematode infested control ($P = 0.05$, n = 10).

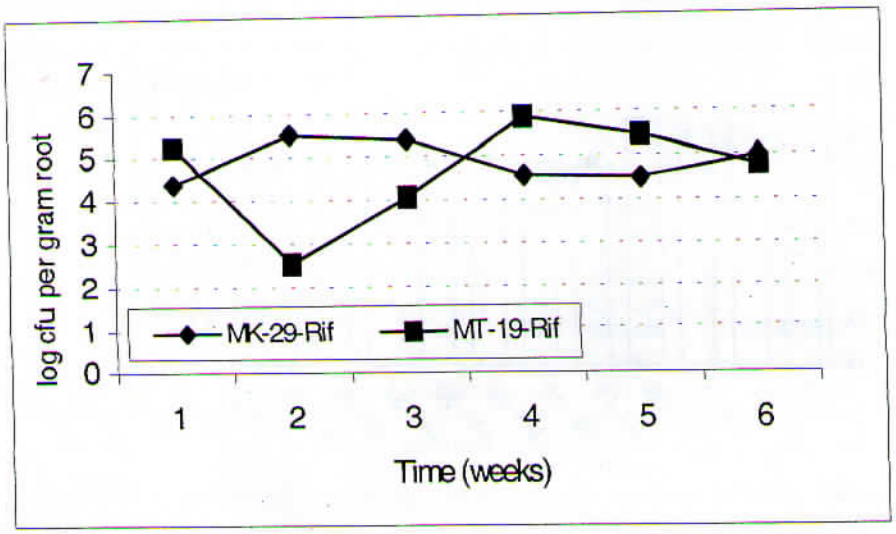

Figure 3. Population dynamics of the 2 endophytic bacterial isolates *Pantoea agglomerans* MK29 and *Pseudomonas putida* MT19 within tomato root tissue over 6 weeks

Internal root colonization. Two rifampicin-resistent strains of endophytic bacteria, *Pantoea agglomerans* MK-29-rif and *Pseudomonas putida* MT-19-rif were tested for their stability of colonization within the plant root over time (Figure 3). Following the soil drench application, the 2

bacteria were detectable in the root plant for over six weeks. The population density over time varied between log 4.4 and 5.5 cfu/g root tissue for strain MK-29-rif and between log 2.5 and 5.9 cfu/g root tissue for strain MT-19-rif.

DISCUSSION

The data obtained from these experiments demonstrated the beneficial effects of endophytic bacteria on both nematode control as well as plant growth. Several isolates of endophytic bacteria were able to increase fresh shoot weight and root length and/or reduced the number of *Meloidogyne* galls. The beneficial effects of endophytic bacteria were similar for both appliation techniques used.

These results are in agreement with previous work on endophytic bacteria reporting plant growth and health promotion on various crops (summarized in HALLMANN *et al.*, 1997). The mechanisms by which endophytic bacteria increase plant growth still needs to be explored. However, these mechanisms might be similar to those reported for rhizosphere bacteria, which include inhibition of deleterious micro-organisms (KLOEPPER & SCHROTH, 1981; VAN PEER & SCHIPPER, 1989), production of plant growth regulating substances, such as ethylene, auxins, or cytokinins (ARSHAD & FRANKENBERGER, 1991) or release of biologically fixed N_2 (BASHAN & HOLGUIN, 1997). The importance of each mechanism needs to be explored for each endophytic isolate seperately.

Besides plant growth promotion, some isolates of endophytic bacteria, namly *Pantoea agglomerans* MK-29, *Pseudomonas putida* MT-19, *Enterobacter intermedius* MK-42 and *Cedecea davisae* MK-30 significantly reduced the number of galls caused by *M. incognita*. Similar results were reported for strains of the genera *Pseudomonas*, *Brevundimons* and *Serratia*, which reduced the number of *Meloidogyne* galls produced on cotton following seed application (HALLMANN *et al.*, 1998). Antagonistic activity against plant parasitic nematodes is a common phenomenon reported for rhizosphere bacteria. Bacterial strains belonging to the genera *Pseudomonas*, *Bacillus*, *Agrobacterium* and others are known to reduce *M. incognita* on white clover and cucumber (BECKER *et al.*, 1988), *Heterodera schachtii* on sugar beet (OOSTENDORP & SIKORA, 1989; NEIPP & BECKER, 1999), *Heterodera glycines* on soybean (KLOEPPER *et al.*, 1992) and *Globodera pallida* on potato (HASKY-GÜNTHER *et al.*, 1998). The mechanisms involved in control of plant parasitic nematodes vary greatly. Some rhizobacteria are known to produce metabolites with nematicidal activity, such as avermectins (STRETTON *et al.*, 1987), 2,4-diacetylphloroglucinol (CRONIN *et al.*, 1997) or volatile compounds such as organic acids, hydrogen sulfide, hydrogen cyanide, and ammonia which are reported to express fungicidal activity (BUCHENAUER, 1999) but also

might effect plant parasitic nematodes. In addition, endophytic bacteria also can induce systemic resistance against plant parasitic nematodes as reported for the rhizosphere bacterium *Rhizobium etli* G12 (REITZ *et al.*, 2000) which was recently found to colonize the plant endophytically (HALLMANN *et al.*, 2000). The close interaction between endophytic bacteria and the plant cell would favor this mechanisms as a preferred choice for biological control strategies.

The root tissue was already colonized by the endophytic bacteria 1 week after application. Rapid root colonization is reported by QUADT-HALLMANN *et al.* (1997) who observed endophytic colonization of cotton roots to occur as soon as 1 hour after bacterial application. Further studies of in planta enzymatic activity by the same authors demonstrated hydrolysis of wall-bound cellulose in the vicinity of endophytic bacteria suggesting active processes associated with bacterial penetration which would explain the rapid time of colonization. In own experiments, population densities of endophytic bacteria were quite stable over time varying between log 4 and log 6 cfu/g root tissue except for one sample (MT-19-rif after 2 weeks). These endophytic population densities observed in tomato are in accordance with those reported by several other authors for a broad spectrum of host plants (summarized by HALLMANN *et al.*, 1997). This would indicate an optimum carrying capacity of endophytic bacteria. If biocontrol procedures could use this carrying capacity primarily for antagonistic endophytic bacteria, a healthy plant should be the consequence.

Two application methods were compared in this study for their suitability for endophytic bacteria. Both techniques allowed endophytic bacteria to promote plant growth and reduce nematode galling. The choice of application technique did not affect the performance of the top 4 endophytic bacteria. However, the choice of application method can be important for other antagonistic endophytes. Seed treatment allows endophytic bacteria to colonize the root immediately after germination and to preoccupy ecological niches otherwise colonized by pathogens. This strategy would especially be suitable for systemic colonizers, which, after gaining entrance, can spread thoughout the entire plant. On the other hand, seed treatment is frequently reported not to support high rhizosphere population densities (BAHME & SCHROTH, 1987; HATZINGER & ALEXANDER, 1994), a fact, that will be detrimental for local colonizers which are not able to colonize young roots. Therefore, local colonizers would probably benefit more from a soil drench applied 2-3 weeks after sowing when the root system has already developed. A soil drench will allow endophytic bacteria to colonize newly formed lateral roots and root tips which otherwise would not be colonized by local endophytic colonizers. The choice of application method is less important for endophytic bacteria inducing plant resistance against pathogen attack, since under these conditions only a certain population density needs to be reached to switch on the plant defense response. Certainly further research

is needed to understand the plant/endophyte interactions and their mode of actions.

In conclusion, results achieved within this study underline the importance of endophytic bacteria for plant growth and health promotion. Overall, endophytic bacteria are promising candidates as biocontrol agents not only against plant pathogenic nematodes, but also against other plant pathogens.

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