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Beneficial effects of *Enterobacter cloacae* and *Pseudomonas mendocina* for biocontrol of *Meloidogyne incognita* with the endospore-forming bacterium *Pasteuria penetrans*

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Summary – Two rhizosphere bacteria, *Enterobacter cloacae* and *Pseudomonas mendocina*, were isolated from the rhizosphere of tomato plants growing in a soil heavily infested with both root-knot nematodes and the parasitoid endospore-forming bacterium *Pasteuria penetrans*. Bacteria *E. cloacae* and *P. mendocina* stimulated plant growth, inhibited the reproduction of the root knot nematode *Meloidogyne incognita*, and increased the attachment of the endospores of *P. penetrans* on the nematodes *in vitro*. *E. cloacae* significantly increased the reproduction of *P. penetrans* in plant roots. Consequently, the introduction of such bacteria in soils, or cultural practices aimed to increase the activity of native strains of these bacteria, could greatly contribute to the efficiency of nematode biocontrol with *P. penetrans*.

Résumé – ***Stimulation de l'activité antagoniste de* Pasteuria penetrans *envers* Meloidogyne incognita *par* Enterobacter cloacae *et* Pseudomonas mendocina** – Deux bactéries rhizosphériques, *Enterobacter cloacae* et *Pseudomonas mendocina*, ont été isolées à partir de la rhizosphère de plants de tomate prélevés dans un sol très infesté par des nématodes à galles et l'actinomycète *Pasteuria penetrans*. Les deux souches bactériennes ont stimulé la croissance de la plante, inhibé le développement du nématode *Meloidogyne incognita* et augmenté *in vitro* l'attachement des spores de *P. penetrans* sur la cuticule des nématodes. *E. cloacae* a significativement stimulé la multiplication de *P. penetrans* dans les racines. En conséquence, l'utilisation de telles bactéries pourrait améliorer de manière importante l'efficacité de *P. penetrans* contre les nématodes du genre *Meloidogyne*.

Keywords: biocontrol, *Enterobacter cloacae*, *Meloidogyne incognita*, *Pasteuria penetrans*, *Pseudomonas mendocina*.

The actinomycete *Pasteuria penetrans* is a Gram-positive endospore-forming bacterium and is an obligate endoparasite of nematodes. It has been shown that this microorganism has potential as a biological control agent (Oostendorp *et al.*, 1991; Zaki & Maqbool, 1992). The physiology of the relationship between *P. penetrans* and root knot nematodes is well documented (Davies *et al.*, 1992; Davies & Danks, 1993; Afolabi *et al.*, 1995) but knowledge about the ecology of this antagonistic association is limited to the effects of soil moisture (Stirling & Wachtel, 1980; Brown & Smart, 1984; Oostendorp *et al.*, 1991) and temperature (Stirling, 1981; Hatz & Dickson, 1992). Some research has been conducted on the effects of abiotic soil factors involved in the availability of the spores of *P. penetrans* for attachment to nematodes (Spaull, 1984; Oostendorp *et al.*, 1990; Singh & Dhawan, 1992; Mateille *et al.*, 1995, 1996). However information about the potential effects of biotic factors which would be able to interact with attachment is only now being demonstrated by Duponnois *et al.* (1997). These authors have shown that the soil microflora could stimulate the attachment of the spores on the juveniles and consequently reduce the invasion of the roots of tomato plants by the root knot nematodes. To study interactions of rhizosphere bacterial isolates with the efficacy of *P. penetrans*, effects of some rhizosphere bacterial strains on attachment of *P. penetrans* spores to the root-knot nematode, *Meloidogyne incognita*, and on the density of *P. penetrans* were studied.

Materials and methods

NEMATODES

A population of *Meloidogyne incognita* was cultured for 2 months on tomato cv. Roma in a heat sterilised soil (140°C, 40 min). Then the tomato roots were harvested, cut into short pieces and placed in a mist chamber (Sein-

horst, 1950) for 1 week to collect second stage juveniles (J2).

Pasteuria penetrans endospores

The endospores of *Pasteuria penetrans* were produced in females of *M. incognita* infecting tomato plants. The females of *M. incognita* were extracted from root galls (Hussey, 1971), washed in distilled water, crushed into Eppendorf tubes with a small pestle to release the spores of *P. penetrans*, and finally centrifugated (15 000 *g*, 15 min). The supernatant was discarded and the pellet was resuspended in 1 ml alcohol 70% for 72 h in the dark at room temperature to obtain spores free of contaminant microflora, and centrifugated (15 000 *g*, 15 min) three times to replace the alcohol with sterile distilled water.

Bacteria

Bacterial strains were isolated from the rhizosphere of tomato plants growing in a field heavily infested with *M. javanica* and *P. penetrans* (20 000 J2 per dm^3 of soil, 80% of infected J2) and characterized by a sandy-clay soil (sand 84.7%; silt 4.1%; clay 11.2%; C/N 7.5; pH_{H_2O} 7.6). Pieces of roots were blended in sterile distilled water using an Ultraturax blender. Serial dilutions of the suspension were plated on 0.3% TSA medium (Tryptic Soy Agar, Difco) and, after 48 h incubation at 25°C in the dark, 43 distinctive colonies were isolated and subcultured on the same medium.

Attachment of *Pasteuria penetrans* spores to J2 of *Meloidogyne incognita*

The 43 bacterial strains were grown in 0.3% tryptic soy broth (Martin, 1975) for 8 days at 25°C. The bacterial suspensions (more than 10^8 cells·ml^{-1}) were centrifugated (2400 *g*, 20 min) and the pellets were resuspended in 0.1 M $MgSO_4$. The spores of *P. penetrans* (100 µl sterile distilled water with 10^5 spores) were incubated with each bacterial strain (1 ml of the $MgSO_4$ suspension) in small glass tubes (7 × 1 cm) for 1 week at 25°C in the dark. Control treatments consisted of *P. penetrans* only. The attachment tests were performed adding 100 J2 of *M. incognita* in 100 µl of distilled water into each tube. The J2 were not surface disinfested and probably have contaminant bacteria. However, there was no carbon or nitrogen source for bacterial growth in the tubes and it could be admitted that the density of contaminant bacteria was insignificant regarding the inoculated bacterial suspension. The microbial suspensions were removed from each tube after 12 h of incubation at 25°C and the numbers of spores of *P. penetrans* per J2 were determined on twenty J2s randomly chosen by examining each J2 under a microscope (×450). There were five replicates per treatment. Data were statistically analysed according to the Man Whitney U test ($P \leqslant 0.05$).

Identification of two bacterial strains

Out of 43 isolates tested, isolates B22 and B23 were chosen for their ability to improve the attachment of the endospores. In order to characterize these two bacterial strains, the choice of a type of API gallery (API System SA, BioMérieux, Lyon, France) was determined with Gram staining and two tests performed on bacterial colonies: action of β-galactosidase (ONPG: Ref 55601, BioMérieux, Lyon, France) and presence of cytochrome oxidase (Ox: Ref 55922, BioMérieux, Lyon, France). The Gram negative isolates were examined using the API 20NE test system (API 2005) which tests the following activities: nitrate reductase (NIT); tryptophanase (TRP); production of acid metabolites from glucose (GLU); arginine dihydrolase (ADH); urease (URE); β-glucosidase (ESC); proteolysis of gelatin (GEL); β-galactosidase (ONPG); use as carbon sources of glucose (GLU), arabinose (ARA), mannose (MNE), mannitol (MAN), N-acetyl-glucosamine (NAG), maltose (MAL), gluconate (GNT), caprate (CAP), adipate (ADI), malate (MLT), citrate (CIT), phenylacetate (PAC), presence of cytochrome oxidase (OX). The Gram positive isolates were examined using the API 50 CHB test system (API 5043) which tests the production of acid metabolites from the carbohydrates glycerol (GLY), erythrol (ERY), D-arabinose (D ARA), L-arabinose (L ARA), ribose (RIB), D-xylose (D XYL), L-xylose (L XYL), adonitol (ADO), β-methyl-D-xyloside (MDX), galactose (GAL), glucose (GLU), fructose (FRU), mannose (MNE), sorbose (SBE), rhamnose (RHA), dulcitol (DUL), inositol (INO), mannitol (MAN), sorbitol (SOR), α-methyl-D-mannoside (MDM), α-methyl-D-glucoside (MDG), N-acetyl glucosamine (NAG), amygdaline (AMY), arbutine (ARB), esculine (ESC), salicine (SAL), cellobiose (CEL), maltose (MAL), lactose (LAC), melibiose (MEL), sucrose (SAC), trehalose (TRE), inuline (INU), melezitose (MLZ), raffinose (RAF), starch (AMD), glycogene (GLG), xylitol (XLT), gentiobiose (GEN), D-turanose (D TUR), D-lyxose (D LYX), D-tagatose (D TAG), D-fucose (D FUC), L-fucose (L FUC), D-arabitol (D AR), L-arabitol (L AR), glu-

conate (GNT), 2 keto-gluconate (2KG), 5 keto-gluconate (5KG).

Reproduction of *Meloidogyne incognita* and spore density of *Pasteuria penetrans* on tomato plants

Two week-old seedlings of tomato cv. Roma were transplanted in 60 cm^3 pots filled with a sandy soil (sand 92.8%; silt 2%; clay 5.2%; pH H_2O 7.1) which was previously autoclaved (140°C, 40 min). One week after transplanting, the plants were inoculated with 5 ml suspensions containing either 0 or 100 J2s of *M. incognita* in distilled water, 5 ml suspensions of each bacterial strain, B22 or B23 (about 10^9 cfu·ml^{-1}), in 0.1 M $MgSO_4$, and 5 ml suspensions of either 0 or 10^5 spores of *P. penetrans* in distilled water. These suspensions were injected by a syringe into a hole near the plant in each pot. The plants which were not inoculated with bacteria received only 5 ml of 0.1 M $MgSO_4$. The plants were randomly placed in a glasshouse (30°C day, 25°C night, 12 h photoperiod) and watered daily without fertilisation. There were eighteen replicates per treatment.

Five of the plants inoculated with *M. incognita* with or without *P. penetrans* and with or without bacteria were uprooted 9 days after inoculation, and the entire root systems stained with acid fuchsin (Byrd *et al.*, 1983) to estimate the number of J2s which penetrated into the roots.

Eight of the plants were uprooted 1 month after inoculation, and the galls with and without egg masses were numbered. Then the roots were cut into short lengths and placed in a mist chamber (Seinhorst, 1950) for 3 weeks to collect J2s from egg hatching. After 5 or 6 days, the number of spores attached on twenty hatched J2s randomly chosen from the mist chamber were determined. Data were statistically analysed according to the Man Whitney U test ($P \leqslant 0.05$). Proportions were transformed by *Arcsin(sqrt)* before analysis. Finally, the oven-dried weights of roots and shoots (1 week at 65°C) were measured. Data were compared using a one-way analysis of variance ($P \leqslant 0.05$).

The five remaining plants were uprooted one month after inoculation. Their roots were cut into short lengths and blended in distilled water. The suspensions were sieved using a bank of sieves. Spores were collected on the finest (0.45 μm). Then, the spores of *P. penetrans* were counted per root system. Data were statistically analysed according to the Man Whitney U test ($P \leqslant 0.05$).

Results

Attachment tests

Among the 43 bacterial strains tested, nine (B5, B11, B22, B23, B24, B32, B33, B34 and B41) increased the percentages of spore-infested J2 and nine (B16, B22, B23, B24, B32, B33, B41, B42, B43) increased the mean number of spores per J2 (Table 1). No bacterial isolates decreased the attachment.

Characterization of the two bacterial strains B22 and B23 (Table 2)

Bacterial strain B22 was gram-positive and, according to the API 50CH test, was identified as *Enterobacter cloacae*. The bacterial strain B23 was gram-negative and, according to the API 20NE test, was identified as *Pseudomonas mendocina*.

Development of *Pasteuria penetrans*

After a 1 month culture of *P. penetrans* on tomato plants, the number of spores extracted from the blended roots was ten times greater when the inoculum of *M. incognita* and *P. penetrans* was supplemented with *E. cloacae* (Fig. 1). The production of spores was not changed when *P. mendocina* was added. When the roots of plants

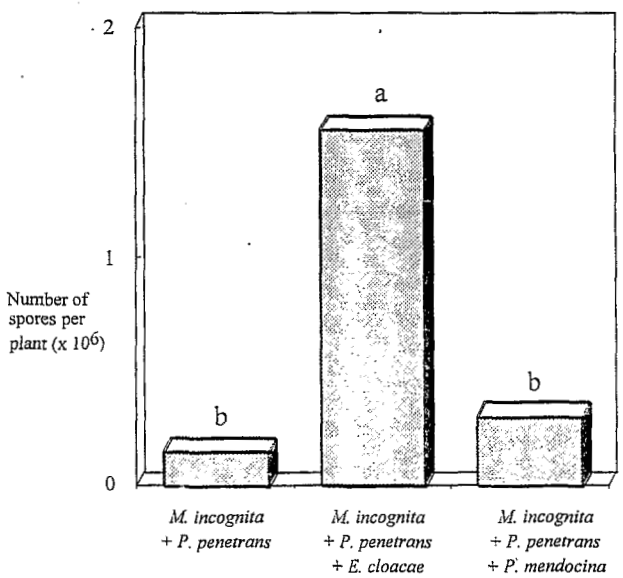

Fig. 1. *Effect of* Enterobacter cloacae *and* Pseudomonas mendocina *on the reproduction of the spores of* Pasteuria penetrans *(Bars indexed by the same letter are not significantly different, $P \leqslant 0.05$).*

Table 1. *Influence of the bacterial strains on the proportions of* Meloidogyne incognita *juveniles infested by* Pasteuria penetrans *and on the number of spores per juvenile*

Bacterial strains	Infested juveniles (%)	Spores per juveniles
Control	32.4	2.31
B1	54.4	2.82
B2	49.3	2.22
B3	45.2	3.00
B4	29.4	2.69
B5	81.4 *	2.69
B6	80.3	1.97
B7	32.6	1.97
B8	29.2	2.55
B9	43.2	2.32
B10	46.1	1.92
B11	84.1 *	3.30
B12	70.4	2.78
B13	57.0	1.95
B14	57.3	2.16
B15	70.3	2.97
B16	71.9	4.29 *
B17	19.9	1.60
B18	16.7	1.47
B19	24.9	2.10
B20	34.2	1.76
B21	55.2	1.87
B22	89.9 *	7.70 *
B23	86.6 *	10.09 *
B24	88.0 *	6.72 *
B25	74.8	3.20
B26	43.7	2.08
B27	39.1	2.25
B28	30.2	1.79
B29	45.2	1.50
B30	42.7	2.99
B31	78.9	4.20
B32	81.8 *	6.44 *
B33	82.3 *	6.39 *
B34	87.1 *	4.76
B35	67.8	4.00
B36	60.6	2.74
B37	47.6	1.76
B38	36.8	1.87
B39	46.1	2.28
B40	39.0	1.46
B41	91.6 *	10.2 *
B42	79.4	6.76 *
B43	77.9	6.35 *

* = significantly different from the control according to Mann Whitney U test ($P \leqslant 0.05$).

Table 2. *Physiological characteristics of the bacterial strains B22 (*Enterobacter cloacae*) and B23 (*Pseudomonas mendocina*) according to the API 50CH and API 20NE tests respectively*

API 50CH test				API 20NE test	
GLY	+	SAL	+	NIT	+
ERY	−	CEL	+	TRP	−
D ARA	−	MAL	+	GLU	−
L ARA	+	LAC	−	ADH	+
RIB	+	MEL	−	URE	−
D XYL	+	SAC	+	ESC	−
L XYL	−	TRE	+	GEL	−
ADO	−	INU	−	ONPG	−
MDX	−	MLZ	−	GLU	+
GAL	+	RAF	−	ARA	−
GLU	+	AMD	−	MNE	−
FRU	+	GLG	−	MAN	−
MNE	+	XLT	−	NAG	−
SBE	−	GEN	+	MAL	−
RHA	+	D TUR	−	GNT	+
DUL	−	D LYX	+	CAP	+
INO	+	D TAG	−	ADI	−
MAN	+	D FUC	−	MLT	+
SOR	+	L FUC	−	CIT	+
MDM	−	D AR	−	PAC	−
MDG	+	L AR	−	OX	+
NAG	+	GNT	+		
AMY	−	2 KG	+		
ARB	+	5 KG	−		
ESC	+				

were placed in the mist chamber, the J2s which hatched had more spores per J2 when *E. cloacae* had been added (Fig. 2).

Development of *Meloidogyne incognita*

Nine days after the inoculation of *M. incognita*, about 33% of the J2 penetrated into the roots, whether or not *P. penetrans* was present (Table 3). When *E. cloacae* or *P. mendocina* were inoculated with the J2 of *M. incognita*, almost all of the J2 penetrated into the roots. When J2 and *E. cloacae* or *P. mendocina* were added, the penetration rate was significantly lower with *P. penetrans* than without *P. penetrans* (56-70 *vs* 96-100%).

One month after the J2 inoculation, the number of root galls was the same with or without *P. penetrans* only, but gall number was greater when other bacteria were added (Table 3). Egg masses were detected among 88% of the galls on the plants inoculated with *M. incognita* alone (Table 3). In the other treatments, the proportion of galls with egg masses was lower when *P. penetrans*

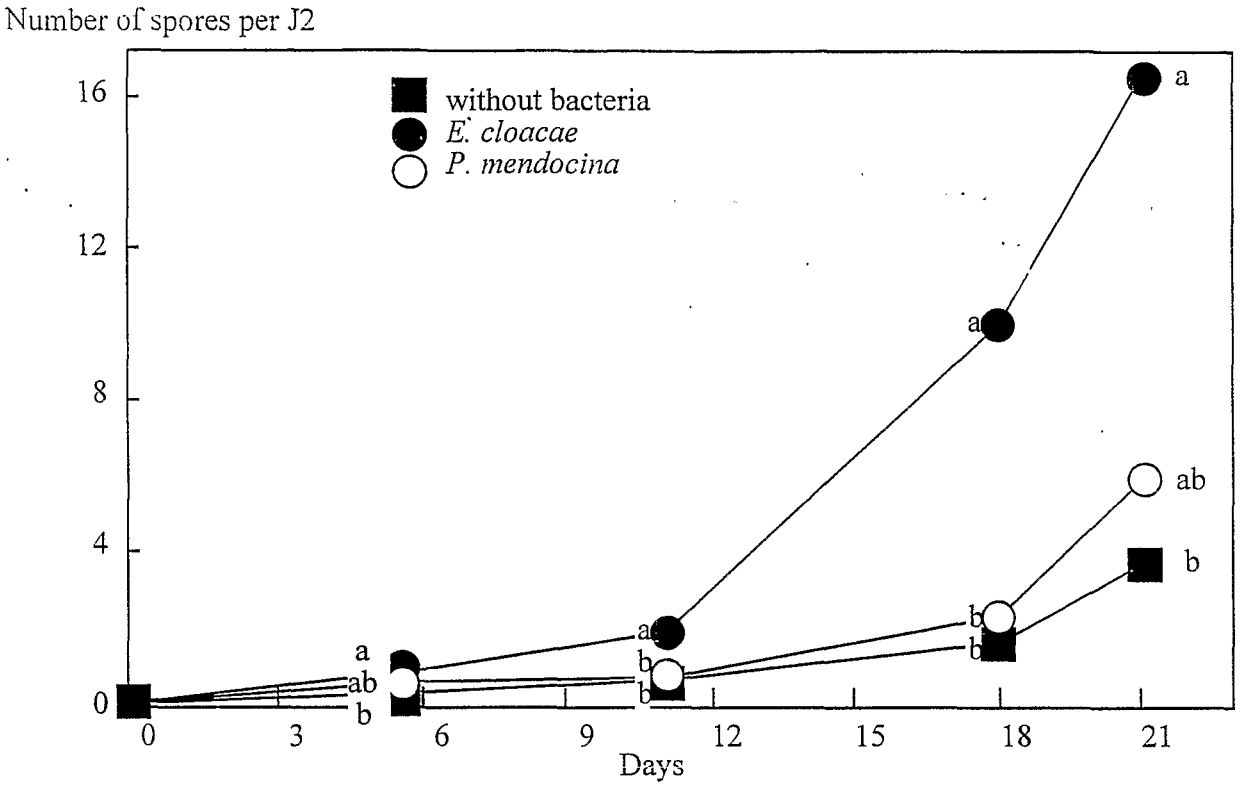

Fig. 2. *Mean number of spores of* Pasteuria penetrans *per J2 of* Meloidogyne incognita *hatched from the roots of the tomato plants co-inoculated with* Enterobacter cloacae *or with* Pseudomonas mendocina *(For each sampling date, data followed by the same letter are not significantly different, $P \leqslant 0.05$).*

Table 3. *Effect of* Enterobacter cloacae *and* Pseudomonas mendocina *inoculated with juveniles of* Meloidogyne incognita *and spores of* Pasteuria penetrans *on the development of the nematodes per plant*

Inoculum	Penetration rate (% of the inoculum)	Number of galls	Number of egg masses	Number of egg masses/number of galls (%)	Number of juveniles
M. incognita	35.1 *d*	33.1 *b*	29.0 *a*	87.6 *a*	10991.0 *a*
M. incognita + *E. cloaceae*	96.0 *ab*	55.9 *a*	20.9 *b*	37.4 *b*	2493.5 *b*
M. incognita + *P. mendocina*	100.0 *a*	58.8 *a*	24.7 *ab*	42.0 *b*	2350.5 *b*
M. incognita + *P. penetrans*	33.1 *d*	40.4 *b*	20.0 *b*	49.5 *b*	6890.2 *ab*
M. incognita + *P. penetrans* + *E. cloaceae*	56.0 *cd*	54.9 *a*	12.8 *c*	23.3 *b*	3500.3 *b*
M. incognita + *P. penetrans* + *P. mendocina*	69.5 *bc*	57.1 *a*	19.3 *b*	33.8 *b*	3692.5 *b*

(Data in the same column followed by the same letter are not significantly different, $P \leqslant 0.05$).

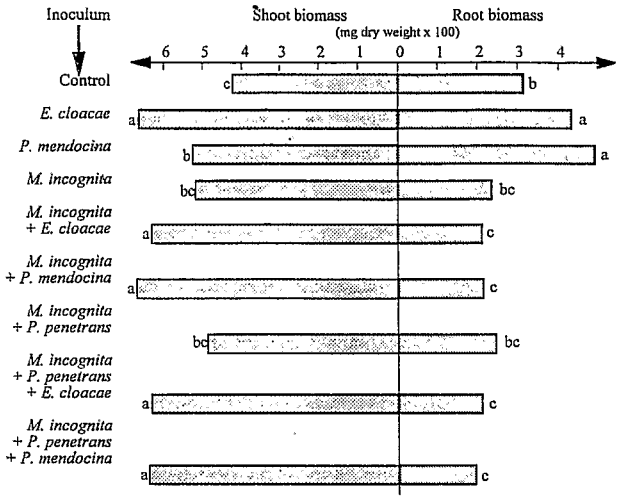

Fig. 3. *Effect of inoculations combining* Meloidogyne incognita, Pasteuria penetrans, Enterobacter cloacae *and* Pseudomonas mendocina *on the plant growth (For each parameter, data followed by the same letter are not significantly different, $P \leqslant 0.05$).*

or the bacteria were added together or separately. Finally, many new generation J2s were recovered from the plants inoculated with *M. incognita* with or without *P. penetrans*, but significantly fewer J2s were recovered from the plants co-inoculated with *E. cloacae* or *P. mendocina* (Table 3).

PLANT GROWTH

There were no significant differences between the growth of the non-inoculated plants (control), the growth of the plants inoculated with *M. incognita* only, and the growth of the plants inoculated with both *M. incognita* and *P. penetrans* (Fig. 3). Plants inoculated with *E. cloacae* or *P. mendocina* only were larger than the control plants. The largest shoot growth was obtained when the plants were inoculated with both *M. incognita* and *E. cloacae* or *P. mendocina*, with or without *P. penetrans*.

Discussion

These results show that some rhizosphere bacteria, including *Enterobacter cloacae* and *Pseudomonas mendocina*, can interact positively with the development of all the components of the life cycle of *P. penetrans*: the host plant, the nematode and *P. penetrans*.

The two bacterial strains have stimulated the growth of the tomato plants. It is well known that some rhizobacteria have a beneficial effect on crop development, which are called plant growth-promoting rhizobacteria or (PGPR) (Kloepper *et al.*, 1989).

This PGPR effect would increase the number of short roots and, consequently, the number of potential sites of penetration for the J2 which would explain the higher penetration rates and the higher number of galls per plant obtained when the bacteria were inoculated. However the number of egg masses which correspond to living females is inhibited by the two bacterial strains. These bacteria induce physiological modifications of the plant roots which interact negatively with the development of the root-knot nematodes. It has been demonstrated recently that the presence of rhizosphere bacteria induce a resistance mechanism toward the potato cyst nematode *Globodera pallida* (Hasky & Sikora, 1995). The fecundity of the females was also reduced. The bacteria could damage the eggshell, principally composed of chitin which is a nitrogenous polysaccharide (polymer of N-acetylglucosamine) and consequently could inhibit the hatching of the juveniles.

The reproduction of *P. penetrans* has two phases: *i)* the attachment of the spores on the cuticle of the nematode, and *ii)* the germination and penetration of the spores into the nematode, its vegetative growth and the sporogenesis (Sayre & Vergin, 1977). In axenic conditions, the two bacterial strains significantly stimulated the spore attachment. The structure of the free spores of *P. penetrans* could be modified by the bacteria. In particular, the sporangial wall and the exosporium could be changed exposing the parasporal fibres and allowing them to make contact with the nematode cuticle.

Although the two bacterial strains stimulated the attachment of the spores on the cuticle of the nematodes, only *E. cloacae* significantly increased the number of *P. penetrans* spores per plant. This bacteria could act on the penetration and the vegetative growth of *P. penetrans* inside the nematode.

E. cloacae can promote the development of *P. penetrans*. Such bacteria would be representative of more promising micro-organisms which have to be considered in order to improve the biocontrol of nematodes with *P. penetrans*. However before the use of this kind of bacteria, the mechanisms must be elucidated in order to provide explanations for these results and to identify more efficient bacteria.

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Nematology

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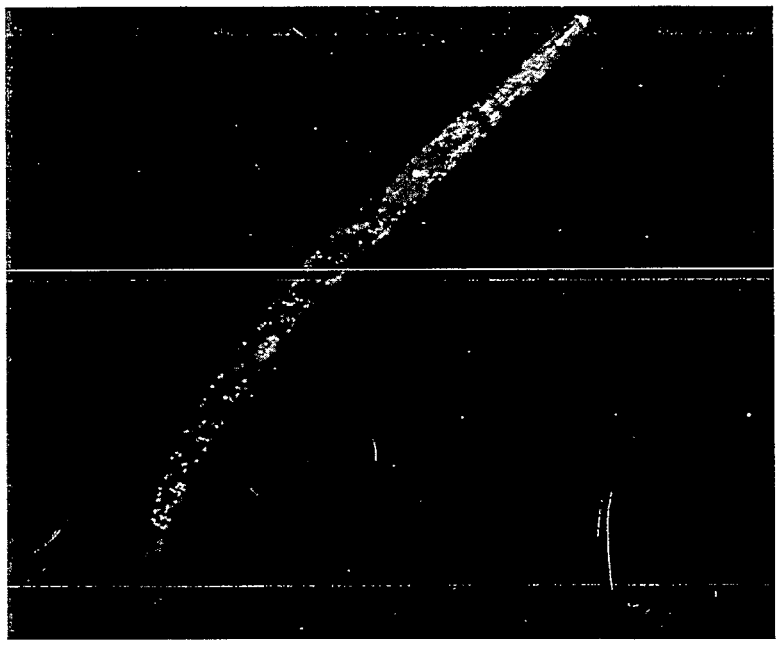

BRILL
LEIDEN · BOSTON · KÖLN

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