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Effects of some rhizosphere bacteria for the biocontrol of nematodes of the genus *Meloidogyne* with *Arthrobotrys oligospora*

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Summary – Some bacteria associated with *Arthrobotrys oligospora* ORS 18692 S7 isolated from a vegetable-producing area in Senegal were tested for their potential effects on fungus development (saprophytic growth and trapping activity), multiplication of *Meloidogyne mayaguensis* on tomato plants, and growth of the host-plant. Three bacterial strains enhanced *in vitro* fungal activity against the nematode, which resulted in better control of the nematode and improved plant growth. These bacteria were called Nematophagous fungus Helper Bacteria (NHB). No criteria could be found for predicting the helper effect of these bacteria. The potentialities of NHB for nematode biocontrol are discussed. © Elsevier - ORSTOM

Résumé – ***Action de bactéries rhizosphériques dans le contrôle biologique des nématodes du genre* Meloidogyne *par* Arthrobotrys oligospora** - A été étudiée l'action d'isolats bactériens associés au champignon nématophage *Arthrobotrys oligospora* ORS 18692 S7 isolé au Sénégal sur le développement du champignon (croissance saprophytique et activité prédatrice), la multiplication d'une population de *Meloidogyne mayaguensis* sur des plants de tomate, et la croissance de la plante hôte. Trois isolats bactériens ont été capables de stimuler *in vitro* l'activité prédatrice du champignon et de réduire le développement d'une population de *M. mayaguensis* sur des plants de tomate cultivés en pots. Ces bactéries ont été nommées bactéries auxiliaires de champignons nématophages (BAN). Les critères permettant de distinguer ces organismes des autres bactéries rhizosphériques n'ont pu être mis en évidence. L'utilisation des BAN pour le contrôle biologique des nématodes est discutée. © Elsevier - ORSTOM

Keywords: *Arthrobotrys oligospora,* biocontrol, helper bacteria, *Meloidogyne,* nematodes, rhizosphere, Senegal, tomato.

Plant-parasitic nematodes, especially root-knot nematodes, are important cosmopolitan pathogens affecting the production of tropical and subtropical crops (Johnson & Fassuliotis, 1984). During the last few decades, nematode control was based mostly on the use of chemicals. However, because of environmental toxicity and cost of these chemicals, other control techniques have been investigated. Besides various biocontrol agents such as vesicular and arbuscular mycorrhizae (Hussey & Roncadori, 1982), nematophagous fungi such as *Verticillium chlamydosporium* (Kerry, 1990; Bourne *et al.*, 1994), rhizobacteria (Racke & Sikora, 1986), and fungal endophytes (Schuster *et al.*, 1995), research focused on nematode-trapping fungi such as *Arthrobotrys irregularis* (Cayrol, 1983; Pelagatti *et al.*, 1986). However, none of these nematode antagonists gave more than 60% control when added to nematode-infested soils (Kerry & Gowen, 1995). Abiotic soil factors (moisture, pH, temperature, organic matter) are known to influence both growth and activity of these microorganisms (Mosse, 1972). However, recent research revealed that such activity is strongly enhanced by associated rhizobacteria (Duponnois *et al.*, 1993; Mateille & Duponnois, 1996).

A survey of *Arthrobotrys* spp. made in all vegetable-producing areas in Senegal (Duponnois *et al.*, 1995) showed that the presence of the fungi was linked to a relatively high organic content and, consequently, to high bacterial populations.

This article presents the potential effects of rhizobacteria on *in vitro* growth and nematode-trapping activity of *Arthrobotrys oligospora*, population development of *Meloidogyne mayaguensis*, and growth of nematode-infected tomato plants.

Materials and methods

Isolation and culturing of *Arthrobotrys oligospora* and associated bacterial strains

Strain ORS 18692 S7 of *Arthrobotrys oligospora* was isolated from sandy soil in a vegetable field in Senegal and cultured on diluted brewery wort using techniques described by Duponnois *et al.* (1995). When bacterial contaminants were detected in some fungal cultures, 5 ml of sterile $MgSO_4$ 0.1 M were poured on the mycelium and the bacteria. Serial dilutions of homogenized bacterial suspensions were plated on 0.3% TSA (Tryptic Soy Broth, DIFCO). Among 120

bacterial isolates, thirteen were randomly chosen (ORS B92S7.1 to ORS B92S7.13). Six other bacteria were also tested: ORS B18690 S2 associated with the mycelium of a nematophagous fungus *Arthrobotrys* species isolated in Senegal (Duponnois *et al.*, 1995), *Pseudomonas mendocina* S23 and *Enterobacter cloacae* S22 isolated from the rhizosphere of tomato roots in Senegal, and three bacterial strains obtained from the Coleccion Espanola de Cultivos Tipo (Universidade de Valencia, Spain): *Pseudomonas striata* CCUG 2525, *Bacillus polymixa* CECT 153, and *Bacillus licheniformis* CECT 20.

Effect of bacterial strains on the *in vitro* development of *Arthrobotrys oligospora* ORS 18692 S7

The fungus was grown in Petri dishes on MNM agar medium (Marx, 1969) at 25°C for 2-3 weeks. Three agar plugs (4 mm thick and 6 mm in diameter) were taken from the margin of each fungal colony and placed in the empty side of two-compartment Petri dishes. The bacteria were cultured on 0.3 % TSA medium in the other side of the dishes. The dishes were sealed to prevent drying and incubated at 25°C in the dark. Two dishes were used for each bacterial strain. Dishes with TSA medium but without bacteria were used as controls. After 48 h incubation, the mean radial growth of the fungus on the dry bottom of the dishes was evaluated as the mean of two measurements taken at 90° of each other, and the number of fructifications per fungal colony was calculated.

Effect of rhizobacteria on the *in vitro* predaceous activity of *Arthrobotrys oligospora* ORS 18692 S7 on nematodes

The bacterial strains were grown in Petri dishes on 0.3 % TSA agar medium at 25°C in the dark for 2-3 days. Five ml of sterile distilled water were poured on each bacterial culture to obtain homogenized bacterial suspensions. The fungal plugs, prepared as described above, were dipped for 1-2 min in the bacterial suspensions, then transferred to Petri dishes filled with distilled water agar (20 $g.L^{-1}$). Fungal plugs dipped in sterile distilled water previously poured on TSA agar medium without bacteria were used as controls. Treatments were replicated five times. The Petri dishes were sealed and incubated at 25°C in the dark.

Two weeks later, 100 7 day-old J2 of *Meloidogyne javanica*, *M. incognita*, or *M. mayaguensis* in 100 µl sterile distilled water were added to the fungal cultures. Populations of *M. javanica*, *M. incognita*, and *M. mayaguensis* were reared on tomato (*Lycopersicon esculentum* Mill.) cv. Roma. Two months after inoculation, roots were cut into 2-3 cm pieces and placed in a mist chamber for 1 week. Nematode eggs hatched and the J2 were collected (Seinhorst, 1950). After 48 h, the J2 trapped by the fungus were counted. The trapping rates (trapped J2/total J2) were transformed by arcsin rate prior to statistical analysis.

Effect of rhizobacteria on the development of nematode populations and of *Arthrobotrys oligospora* in soil

The fungal strain *A. oligospora* S18692 S7 was grown in glass flasks in liquid MNM medium (Marx, 1969) for 2 weeks at 25°C. The fungal suspension was then centrifuged (2400 × *g*, 30 min) and the supernatant was eliminated. The pellet was resuspended three times in $MgSO_4$ 0.1 M and centrifuged (2400 × *g*, 30 min) to eliminate the culture medium; it was finally suspended in $MgSO_4$ 0.1 M.

The bacterial isolates with the best *in vitro* help effect during the previous experiment were cultured in 3 $g.l^{-1}$ liquid Difco tryptic soy broth in glass flasks shaken for 8 days at 25°C. The bacterial suspensions were then centrifuged (2400 × *g*, 10 min) and the pellet resuspended in $MgSO_4$ 0.1 M.

Tomato seedlings were transplanted in 60 ml polythene pots filled with autoclaved (140°C, 40 min) sandy soil (clay, 3.9 %; silt, 2.9%; sand, 92.2 %; carbon, 3.7 %; nitrogen, 0.45 %; $pH_{[H_2O]}$, 8.3).

Two weeks after transplantation, 1 ml of the fungal suspension (1 mg dry weight of fungal biomass) and 5 ml of each bacterial suspension (about 10^{12} colony forming units [cfu].ml^{-1}) were injected with a syringe into each tomato pot. Inoculation with $MgSO_4$ 0.1 M without any fungi or bacteria was used as a control. One week later, half of the tomato seedlings were inoculated with 5 ml suspensions containing 100 7-day-old *M. mayaguensis* J2. The rest of the plants were watered with 5 ml of distilled water and used as control. Each nematode-fungus-bacteria combination was replicated ten times.

The seedlings were harvested one month after nematode inoculation. Shoots were dried at 65°C for 1 week and weighed. The roots were washed, cut into 1-2 cm pieces, and left in a mist chamber for 2 weeks for the recovery of hatched J2 (Seinhorst, 1950). The numbers of J2 were *log (x + 1)* transformed for statistical analysis. The roots were oven dried and weighed. A 1 g soil subsample was taken from each pot and incubated for one week in Petri dishes on diluted brewery wort (1.8-2.0 $g.L^{-1}$ total sugars after dilution, pH 5.5) at 25°C in the dark. *A. oligospora* conidiophores were counted to evaluate the presence of the fungus in the soil.

Statistical analysis

All the data were subjected to a one-way analysis of variance and the mean values were compared with the Student's t-test ($P \leq 0.05$).

Results

Effect of the bacterial strains on the *in vitro* development of *Arthrobotrys oligospora*

Nine bacterial strains – *Enterobacter cloacae* S22, *Bacillus licheniformis,* and seven strains isolated from *A. oligospora* ORS 18692 S7 conidiophores – significantly decreased *in vitro* radial growth of the fungus (Fig. 1). Three bacterial strains – *E. cloacae* S22, *B. licheniformis,* and strain B 92S7.1 – significantly reduced the number of conidiophores per fungal colony (Fig. 2). Most of the other strains increased this number. For each bacterial strain, the number of fructifications per fungal colony was significantly correlated with radial growth of the fungus (Fig. 3).

Effect of the bacterial strains on the *in vitro* trapping activity of *A. oligospora* ORS 18692 S7 on *Meloidogyne* juveniles

Among the bacteria identified, *P. mendocina* S23, *B. polymixa,* and *B. licheniformis* stimulated the trapping activity of *A. oligospora* ORS 18692 S7 on *M. mayaguensis* J2 (Table 1). *B. polymixa* was also effective on *M. javanica*. *Enterobacter cloacae* and *P. striata* enhanced the activity of the fungus on *M. incognita* and *M. javanica,* respectively. Among the bacteria unidentified, the strains B 92S7.1, B 92S7.9, B 92S7.10, and B 92S7.12 were effective on the three *Meloidogyne* species. Others, such as B 92S7.2, B 92S7.8 and B 92S7.11, were effective on two of the *Meloidogyne* species. Most often one of these species was *M. incognita*.

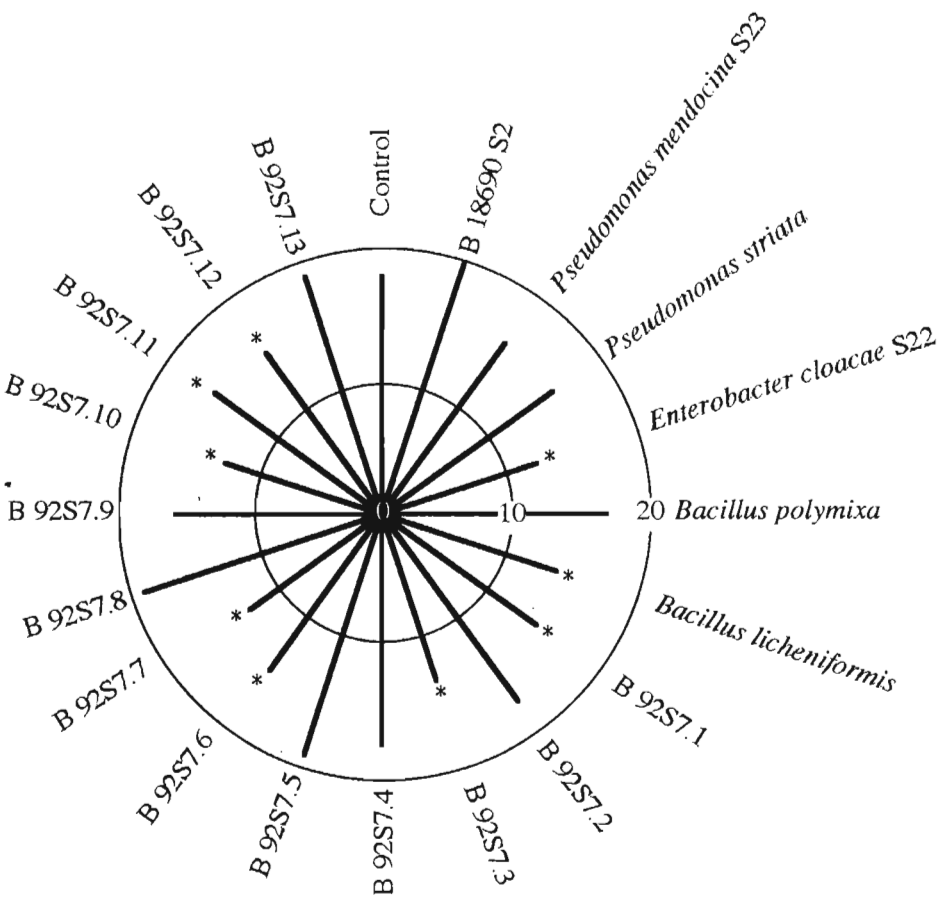

Fig. 1. In vitro *effect of rhizosphere bacteria on the radial growth (mm) of* Arthrobotrys oligospora *ORS 18692 S7 (data followed by an asterisk were significantly different from the control [no bacteria], according to a one-way analysis of variance* $P \leq 0.05$*).*

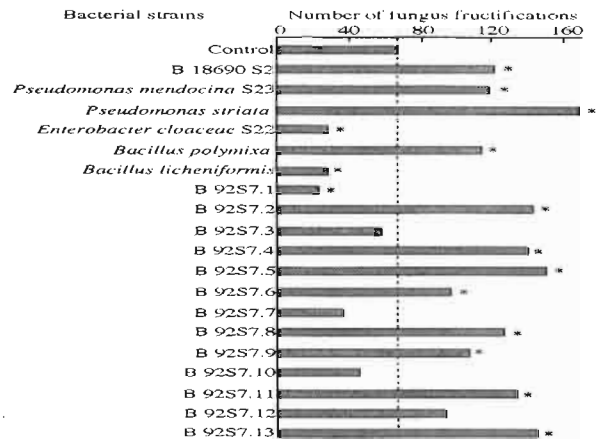

Fig. 2. In vitro *effect of rhizosphere bacteria on the number of fructifications per fungal colony of* Arthrobotrys oligospora *ORS 18692 S7(data followed by an asterisk were significantly different from the control [no bacteria], according to a one - way analysis of variance,* $P \leq 0.05$).

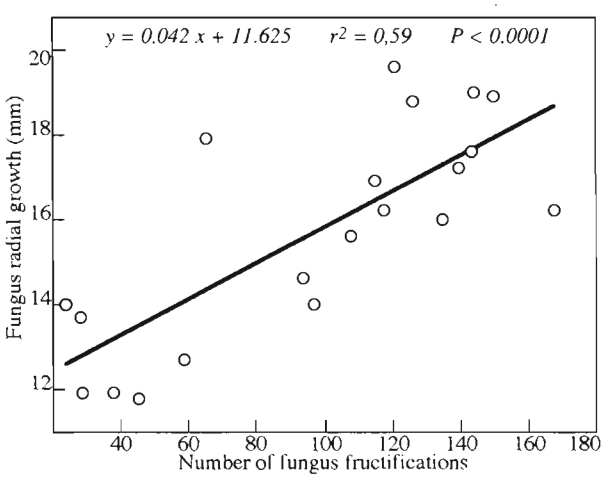

Fig. 3. *Relation between the number of fructifications per fungal colony and the radial growth of* Arthrobotrys oligospora *ORS 18692 S7.*

Effect of the bacterials strains on the multiplication of *M. mayaguensis* on tomato plants inoculated or not with the fungus (Table 2)

Without *A. oligospora*, the total population of *M. mayaguensis* extracted from roots and soil was significantly higher when the bacterial strains *E. cloacae* and B 92S7.9 were added to the soil. The other bacteria had no effect. With *A. oligospora*, the population of *M. mayaguensis* was higher when *E. cloacae* was added to the soil, but it was reduced by half when the bacterial strains B 92S7.1, B 92S7.7, and B 92S7.12 were added.

Effect of rhizobacteria on the development of *Arthrobotrys oligospora* in the soil (Table 3)

Five weeks after the fungus was inoculated into soil, the number of *A. oligospora* conidiophores was significantly higher in soil supplemented with the bacterial strains B 92S7.3, B 92S7.8, B 92S7.9, and B 92S7.12 in the absence of nematodes. When the nematodes were inoculated to the tomato plants, the number of conidiophores increased only with bacterial strains B 92S7.8 and B 92S7.12. In some cases (no bacteria, *B. licheniformis*, strains B 92S7.1, B 92S7.8, and B 92S7.12), the development of the fungus increased when the plants were inoculated with nematodes.

Effect of nematodes, fungi, and bacterial strains on growth of tomato plants (Table 4)

The growth of both shoots and roots of plants was significantly higher when *P. mendocina* S23 was inocu-

Table 1. *Effect of the bacterial strains on the trapping activity (percentage of trapped J2) of* Arthrobotrys oligospora *ORS 18692 S7 on* Meloidogyne mayaguensis, M. javanica, *and* M. incognita *(data followed by an asterisk were significantly different from the control (no bacteria), according to a one - way analysis of variance,* $P \leq 0.05$).

Bacterial strains	Trapping activity		
	M. mayaguensis	*M. javanica*	*M. incognita*
Control	12	2	57
B 18690 S2	41	56 *	53
Pseudomonas mendocina S23	53 *	11	79
Pseudomonas striata	24	67 *	67
Enterobacter cloacae S22	29	10	95 *
Bacillus polymixa	60 *	34 *	73
Bacillus licheniformis	57 *	4	40
B 92S7.1	59 *	68 *	92 *
B 92S7.2	31	56 *	80 *
B 92S7.3	39	27	39
B 92S7.4	3	0	78
B 92S7.5	20	11	95 *
B 92S7.6	23	7	89 *
B 92S7.7	58 *	9	74
B 92S7.8	73 *	27	84 *
B 92S7.9	56 *	40 *	92 *
B 92S7.10	74 *	40 *	93 *
B 92S7.11	23	37 *	97 *
B 92S7.12	85 *	43 *	96 *
B 92S7.13	13	20	69

Table 2. *Effect of the bacterial strains on the numbers of J2 of* Meloidogyne mayaguensis *per tomato plants inoculated or not with* Arthrobotrys oligospora *ORS 18692 S7 (data followed by an asterisk were significantly different from the control [no bacteria], according to a one-way analysis of variance,* $P \leq 0.05$).

Bacterial strains	Number of J2 per plant	
	without *A. oligospora*	with *A. oligospora*
Control	4709	5026
B 18690 S2	4686	4399
Pseudomonas mendocina S23	4389	6911
Enterobacter cloacae S22	8346 *	7879 *
Bacillus licheniformis	5756	3234
B 92S7.1	6579	2527 *
B 92S7.3	5637	3164
B 92S7.7	4493	2683 *
B 92S7.8	5899	4629
B 92S7.9	8300 *	5367
B 92S7.10	3651	3415
B 92S7.12	4114	2171 *

Table 3. *Development of* Arthrobotrys oligospora *ORS 18692 S7 in the soil according to the bacterial strains inoculated and the presence of* Meloidogyne mayaguensis *(data followed by an asterisk were significantly different from the control [no bacteria], according to a one-way analysis of variance* ($P \leq 0.05$).

Bacterial strains	Number of conidiophores per g of soil (dry weight])	
	without *M. mayaguensis*	with *M. mayaguensis*
Control	10.6	22.1
B 18690 S2	2.2	5.8
Pseudomonas mendocina S23	10.4	14.7
Enterobacter cloacae S22	25.9	22.7
Bacillus licheniformis	3.1	12.7
B 92S7.1	20.8	31.3
B 92S7.3	29.4 *	32.9
B 92S7.7	16.0	17.5
B 92S7.8	40.1 *	54.6 *
B 92S7.9	56.0 *	21.2
B 92S7.10	22.4	38.2
B 92S7.12	35.1 *	63.9 *

lated, regardless of the presence of the fungus and the nematodes. Shoot and root biomasses increased when the bacterial strains B 92S7.1, B 92S7.7, or B 92S7.8 were inoculated alone. When nematodes were added, the bacterial effects were negated except for bacterial strains B 18690 S2 and B 92S7.9, which increased shoot and root biomass. When the fungus was inoculated without nematodes, shoot growth increased with most of the bacterial strains. When it was inoculated with nematodes, shoot biomass increased only with *B. licheniformis* and B 92S7.3, and root biomass only with B 92S7.7 and B 92S7.9.

Discussion

Root exudates provide most of the low molecular weight compounds that are easily available for microorganisms. This is a reason why microbial populations are larger and more diversified in the rhizosphere than in soil. All of the rhizosphere microorganisms interact with each other, especially the bacteria that stimulate either symbiotic or antagonistic biological processes. For example, the effects of bacteria on the establishment of ectomycorrhizal associations have been well studied (Garbaye & Bowen, 1989; Garbaye *et al.*, 1990; Duponnois & Garbaye, 1991, 1992; Duponnois, 1992; Garbaye & Duponnois, 1992; Duponnois *et al.*, 1993.) The bacteria isolated from mycorrhizae and sporocarps of *Laccaria laccata* Scop. ex. Fr. and Douglas fir (*Pseudotsuga menziesii* Mirb. Franco) have multiple effects, including the stimulation of fungal growth by some unidentified volatile compounds. For this reason they are called Mycorrhization Helper Bacteria (MHB).

The main topic of this research on some nematophagous fungus-bacterium interactions was to identify bacterial isolates that could enhance the trapping activity of the fungal strain *A. oligospora* ORS 18692 S7 against the root-knot nematode *M. mayaguensis*. The same experimental approach as used for MHB was followed. Some bacteria present in the vicinity of nematophagous fungi were isolated and tested for their capacity to improve the activity of the fungus, to decrease the multiplication of the nematode population, and therefore to improve plant growth.

Only inhibiting effects by some bacterial strains on the fungal radial growth have been recorded by gaseous way. Fungal growth was positively correlated with the number of conidiophores per fungal colony. Here, carbon dioxide could have played an important role. Depending on its concentration, it decreases or increases the growth of various fungi (Imolehin & Grogan, 1980 ; Straastma *et al.*, 1986). However, other compounds can be involved such as ethylene (Imolehin & Grogan, 1980), ammonia, amines, alcohols, sulphur compounds, or low-molecular weight fatty acids (Duponnois, 1992).

Specific biochemical (lectins) mechanisms manage the relationships between nematophagous fungi and *Meloidogyne* species (Nordbring-Hertz & Mattiasson, 1979; Imerglik, 1981). In this study, *M. mayaguensis* and *M. incognita* were better targets of the fungal

Table 4. *Effects of the bacterial strains on the growth of the tomato plants in the absence (-) or presence (+) of* Meloidogyne mayaguensis *and* Arthrobotrys oligospora *(data followed by an asterisk were significantly different from the control (no bacteria), according to a one-way analysis of variance,* $P \leq 0.05$*; nd = not determined).*

	Shoot biomass (mg dry weight)				Root biomass (mg dry weight)			
A. oligospora	-	-	+	+	-	-	+	+
M. mayaguensis	-	+	-	+	-	+	-	+
Bacterial strains								
Control	119.0	153.0	94.0	123.0	99.0	100.0	90.0	83.0
B 18690 S2	117.0	187.0 *	139.0 *	176.0 *	101.0	105.0	113.0	113.0
Pseudomonas mendocina S23	155.0 *	197.0 *	155.0 *	165.0 *	177.0 *	131.0 *	199.0 *	130.0 *
Enterobacter cloacae S22	138.0	176.0	102.0	141.0	134.0	99.0	86.0	84.0
Bacillus licheniformis	116.0	143.0	111.1	156.0 *	132.0	78.0	86.7	106.7
B 92S7.1	153.0 *	144.0	129.0 *	145.0	122.0	87.0	112.0	93.0
B 92S7.3	128.0	128.0	147.0 *	169.0 *	nd	105.0	123.0	82.0
B 92S7.7	151.8 *	157.3	121.0 *	136.0	131.8	103.6	113.0	115.0
B 92S7.8	117.8	141.1	112.2	116.7	143.3 *	113.3	120.0	90.0
B 92S7.9	114.0	119.0	131.1 *	131.1	89.0	62.0 *	184.4 *	155.6 *
B 92S7.10	132.0	123.0	133.0 *	126.0	131.0	78.0	131.0 *	76.0
B 92S7.12	113.0	133.0	103.0	141.0	99.0	96.0	88.0	75.0

strain *A. oligospora* ORS 18692 S7 than *M. javanica*. However, when the J2 were in presence of fungi combined with bacteria, this specificity did not appear with the bacterial strains B92S7.1, B92S7.9, B92S7.10, and B92S7.12 and the aggressiveness of the fungus increased. The effects of these bacterial strains on nematodes can be explained by the production of exopolysaccharides or toxic compounds, which would reduce the motility of the juveniles (Sikora, 1988), or by the production of oligosaccharides or simple sugars (Rozycki, 1987), which could act as molecular bridges between the two microorganisms and thus widen the specificity of the relationships between fungus and *Meloidogyne* species.

Some bacterial strains, such as Plant Growth Promoting Rhizobacteria (Kloepper *et al.*, 1980), also are able to stimulate plant growth but none of them inhibited multiplication of *M. mayaguensis*. However, with dual inocula, three bacterial strains (B 92S7.1, B 92S7.7, and B 92S7.12) enhanced the activity of the fungus. The multiple effects of these bacteria were summarized (Table 5), but no taxonomic and metabolic characteristics could be defined to distinguish these bacteria from other rhizosphere bacteria and to predict their helper effects on the predatory activity of nematophagous fungi.

Some bacteria isolated from the nematophagous fungus *A. oligospora* act as helpers of the fungal activity and for this reason were called NHB (Nematophagous fungus Helper Bacteria). The taxonomic position of these bacteria and the mechanisms involved in the helper function need to be investigated.

Table 5. *Potential effects of three bacterial strains (+ = stimulation; - = inhibition; 0 = no effect).*

Potential effects	Bacterial strains		
	B 92S7.1	B 92S7.7	B 92S7.12
Fungal growth *in vitro*	-	-	-
Fungal fructification *in vitro*	-	0	0
Fungal fructification *in situ*	0	0	+
Trapping activity against *M. mayaguensis*	+	+	+
Specificity of the trapping activity	-	0	-
Plant growth (shoot biomass)	+	+	0
Multiplication of *M. mayaguensis*	-	-	-

From a practical point of view, NHB could be used for nematode biocontrol. It is well known that the production of spores by nematophagous fungi is very limited (Cayrol, 1988). Consequently, these fungi can be used only on a solid substrate humidified with a nutritive medium and easily colonized by the fungal mycelium. However, relatively large quantities of fungal inoculum must be added to the soil to significantly decrease nematode populations. NHB could reduce the required quantities of fungal inocula. They could also extend the host range of these fungi against *Meloidogyne* species.

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