THE NEMATOPHAGOUS FUNGI HELPER BACTERIA (NHB) : A NEW DIMENSION FOR THE BIOLOGICAL CONTROL OF ROOT KNOT NEMATODES BY TRAPPING FUNGI

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SUMMARY

The research work presented in this paper summaries the studies performed in the Biopedology laboratory of IRD Dakar on the biological control with nematophagous fungi against the root-knot nematodes. The results show that the fungi can act against M. incognita and M. mayaguensis, the most infective species in West Africa but also frequently detected through coffee plantations. They can also control the development of Meloidogyne populations on tomato plants. A cultural practice has been developped using compost blocks in which the fungus was inoculated. In a field experiment, the growth of tomato plants has been increased while the nematode development has been decreased. However, all these experiments have been performed during a short period. In order to enhance the competitivity of the fungus, the fungus can be associated with bacterial isolates, called NHB (Nematophagous Helper Bacteria), which stimulate the fungal activity against Meloidogyne but involve also a depressive effect on other plant parasitic nematodes such as Rotylenchulus reniformis.

INTRODUCTION

Many genera and species of plant parasitic nematodes are associated with coffee in the world being therefore responsible for great financial losses to the coffee farmers. Among these pathogens, root-knot nematodes (Meloidogyne spp.) are the most abundant group (Table 1) and the most common species are M. exigua, M. incognita and M. coffeicola. The symptoms of damage due to these pathogens are typical rounded galls on the root systems, white to yellowish brown becoming dark brown in aging roots. The infested coffee plants show foliar chlorosis, leaf fall, general decline. Their growth is generally reduced and sometimes the plants died (Hutton et al., 1982 ; Lordello, 1984). The coffee plantations can be dramatically affected by the nematodes. For example, in Sao Paulo state (Brazil), they have been destroyed by M. incognita with 5-year-old coffee plantations dying out (Lordello, 1984).

The control of nematodes is more difficult in a perennial crop than in annual or herbaceous crops. For example, the rotation schemes, successfully used with annual crops, are impractible with these long term cultures. Moreover surviving roots of excised plants or old plants left in the field can provide nutrients for nematodes and consequently maintain the nematodes population in the soil.

The control practices used by many farmers is generally based on (i) the production of seedlings without root-knot nematodes (i.e. disinfection of nursery soils), (ii) the application of nematicides and/or the culture of resistant or tolerant cultivars. Among these control measures, pesticide compounds are mostly applied to reduce nematode multiplication.

Table 1. Nematophagous fungi used in the experiments

Identification	Code	Geographical origin	Author
Arthrobotrys oligospora	S 30	Burkina Faso	Sawadogo A. (1)
Arthrobotrys oligospora	S 31	Burkina Faso	Sawadogo A.
Arthrobotrys conoides	S 42	Burkina Faso	Sawadogo A.
Arthrobotrys sp.	BF 10	Burkina Faso	Sawadogo A.
Arthrobotrys sp.	BF 74	Burkina Faso	Sawadogo A.
Arthrobotrys sp.	BF 80	Burkina Faso	Sawadogo A.
Arthrobotrys sp.	SOSU 2	Burkina Faso	Sawadogo A.
Arthrobotrys sp.	ORS 18690 S2	Senegal	R. Duponnois
Arthrobotrys oligospora	ORS 18692 S5	Senegal	R. Duponnois
Arthrobotrys oligospora	ORS 18692 S7	Senegal	R. Duponnois

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However they are very expensive and very toxic when improperly. Thus other control techniques such as biological control have been investigated. Besides various microorganisms tested against Meloidogyne spp. such as arbuscular mycorrhizae (Hussey & Roncadori, 1982), eggs parasitic fungi (Verticillium chlamydosporium)(Kerry, 1990 ; Bourne et al., 1994), rhizobacteria (Racke & Sikora, 1986) and fungal endophytes (Schuster et al., 1995), research focused on nematophagous fungi (Cayrol , 1983 ; Pelagatti et al., 1986 ; Duponnois et al., 1996). The screening of efficient fungal strains against Meloidogyne was based on the use of tests performed in axenic or controlled conditions (in vitro tests or glasshouse experiments with desinfected substrates). When these microorganisms were transferred into the field, their antagonistic activity was often modulated (generally decreased) by the environmental conditions. In fact, the topics of this kind of research must integrated the requested qualities of the fungal strain but also its repply from interactions with the soil microbial community in the soil. In the laboratory of biopedology (IRD, Dakar), we have developped a scientific program based on (i) the screening of efficient fungal strains for trapping juveniles of Meloidogyne, (ii) the determination of potential effects of rhizobacteria on in vitro growth and nematode-trapping activity of Arthrobotrys oligospora, population development of plant parasitic nematodes and (iii) develop a control method with the fungal strain incorporated into compost blocks. Tobacco and tomato plants, very susceptible to Meloidogyne, were used in these experiments.

MATERIALS & METHODS

1/ Test of the potential of nematophagous fungi against Meloidogyne

A collection of nematophagous fungi isolated from several vegetable-producing areas in Senegal and Burkina Faso were maintained aseptically in the dark at 25°C on the nutrient broth (8 $g.l^{-1}$) agar (20 $g.l^{-1}$) medium (Table 1). The trapping activity of each fungal strain was estimated using technique described by Duponnois et al. (1996). Populations of different species of Meloidogyne (M. mayaguensis, M. javanica and M. incognita) were reared on tomato (Lycopersicon esculentum Mill.), cv Roma roots. Two months after inoculation, the roots were harvested, cut into short pieces and placed in a mist chamber for one week for egg hatching (Seinhorst, 1950). Fungal agar plugs were taken from the margin of two week old colonies and transferred into Petri dishes filled with distilled water agar (20 $g.l^{-1}$). One week later, 100 second stage juveniles suspended in a water droplet were placed on these fungal cultures. After two days, the number of juveniles trapped by the fungus were counted under a dissecting microscope. Each combination Meloidogyne species-fungal isolate was replicated five times. The trapping rate (trapped juveniles / total juveniles) was transformed by arcsin(sqrt) and treated with a one way analysis of variance, the mean values being compared with the Student's t-test at 0.05 probability level.

The ability of these fungal strains to control Meloidogyne populations was examined through a glasshouse experiment. Solid fungus inocula were prepared in 0.5 dm^3 glass flasks containing 0.3 dm^3 compost. After autoclaving at 120°C for 40 min, the substrate was moistened to field capacity with liquid nutrient broth medium (8 $g.l^{-1}$), the jars closed with cotton wool and autoclaved a second time at 120°C for 20 min. After cooling, mycelial plugs, sampling from each fungal cultures on Petri dishes, were put into each glass flasks. The cultures were incubated for 5 weeks at 25°C.

The fungal inoculum was mixed to an autoclaved (140°C, 40 min) sandy soil (pH H_2O 7.1 ; fine silt 0.6% ; coarse silt 1.4% ; fine sand 61.6%) at the rate 1 : 100 (v :v). This mixture was divided in 60 cm^3 polythene cells (The control treatment received the same quantity of compost but without fungus). Then a one week old tomato seedling was transferred into each cell. After 1 week, the tomato plants were inoculated with 5 ml suspension of 100 7-day-old second stage juveniles of M. mayaguensis (water alone for the control). The cells were placed in a glasshouse under natural climatic conditions (temperatures from 20°C to 35°C ; about 15 hours light per day). The treatments were watered daily and arranged in a randomized complete block design with 14 replicates. One month after the nematode inoculation, the plants were uprooted and the roots washed. Shoots were dried at 65°C for one week and weighed. Galls induced by M. mayaguensis were counted. Then roots were cut into 1 to 2 cm pieces and placed in a mist chamber for 2 weeks to recover hatched juveniles (Seinhorst, 1950). Roots were then oven-dried and weighed. The data were treated with an one way analysis of variance and the mean values were compared with the Student's t test

($P < 0.05$). For nematodes, data were previously transformed by log (x+1). 2/ Study of the potential effects of **rhizobacteria** on the antagonistic activity of Arthrobotrys oligospora against Meloidogyne spp.

The basis of this research program have been described in Duponnois & Bâ (1998). The authors have demonstrated that a large nematophagous fungus population was associated with the presence of bacteria belonging to the group of Pseudomonas fluorescent. Furthermore, the aims of this research were to test the effect of these bacteria on the fungal growth (a strain of A. oligospora T41), on its predatory activity and to evaluate the bacterial influence on the fungal control of the multiplication of Meloidogyne population.

Bacterial strains were cultured in Petri dishes on 0.3% TSB agar medium at 25°C for two days. The bacterial cultures were suspended in 5 ml of sterile magnesium sulfate solution (0.1 M) with a bend glass rod. The control treatment was prepared in the same way from a Petri dish containing the same agar medium without bacteria. One set of experiment was performed by direct liquid contact between the mycelium and the bacteria. Fungal plugs sampled as described above were dipped in the bacterial suspensions or in the control solution for 1-2 min and transferred into empty Petri dishes (Duponnois & Garbaye, 1990). Another set of expriment was done with no liquid contact. Two-compartment dishes were used: the fungal plugs were laid on the dry bottom of the dish for one compartment while the other was filled by 0.3% TSB agar medium inoculated or not (control treatment) by the bacterial strains.The gas diffusion from one side to the other was permitted because the wall separating the two compartments did not touch the lid of the dish.

In both experiment types, two dishes, each with 3 mycelial plugs, were prepared for each treatment. Then the dishes were incubated at 25°C for four days. Observation and numeration were carried out through the lid with a stereomicroscope and the mean radial growth in two perpendicular direction was calculated. The data were statistically compared to the control treatments without bacteria with the Student "t" test ($P<0.05$).

The fungal plugs, dipped in the bacterial suspensions or control treatment as described above, were transferred to Petri dishes filled with distilled water agar (20 $g.l^{-1}$). There were five replicates per treatment. The Petri dishes were incubated at 25 °C in the dark. Two weeks later, 100 7-day-old-second stage J2 of Meloidogyne mayaguensis suspended in 100 µl sterile distilled water were placed on the fungal cultures. Populations of 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 one week to allow nematode eggs to hatch and the juveniles to be collected (J2) (Seinhorst, 1950). After 48 hours, the number of the juveniles trapped by the fungus were counted. The data were statistically treated as described above.

Tobacco (Nicotiana tabacum L. var. Paraguay x Claro) seedlings were grown in 60 ml polythene pots filled with an autoclaved soil (140 °C, 40 min) sandy soil (clay : 3.9 %; silt : 2.9%; sand : 92.2 %; carbon : 3.7 %; nitrogen : 0.45 %; pH(H_20) : 8.3) inoculated with 1 mg dry weight of fungal biomass and/or 5 ml of each bacterial suspension (about 10^{12} colony forming unit $(cfu).ml^{-1}$). The fungal strain had been grown in one litre glass flask filled with 0.5 litre of 0.3% TSB medium for 2 weeks at 25 °C. The fungal suspension was then filtered and the mycelium was collected. The fungal mycelium was three times washed in $MgSO_4$ 0.1 M and finally suspended in $MgSO_4$ 0.1 M. The bacterial isolates 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. The control treatments were performed by injecting in the soil 1 ml of $MgSO_4$ 0.1 M without either fungus or bacteria. There were ten replicates per treatment.

After 2 month culture, 10 tobacco plants from each treatment were transferred in 10 litre pots filled with the same soil as above but non autoclaved. The pots were placed in a glasshouse under natural conditions (temperatures ranged from 20°C to 35°C ; about 15 hours light per 24 h). After two month culture, the plants were harvested and the root systems gently washed. The soil from each pot was mixed, a 250 g sub-sample was taken and the nematodes were extracted by the Seinhorst's (1962) elutriation technique. The oven-dried weight of shoot (one week at 65°C) was measured. Each root system was then cut into 2-3 cm pieces and placed in a mist chamber for two weeks in order to recover nematodes (Seinhorst, 1950). The nematodes were counted

under a stereomicroscope (magnification x 150). The means of treatment vs. control (not inoculated) were compared with Student's 't' test at 0.05 probability level. For the nematode populations, data were transformed by log (x + 1) prior to analysis.

3/ Development of a control method with the fungal strain incorporated into compost blocks

The fungal inoculum (strain ORS 18692S7) was prepared using the compost as describe in the section 1/. Then it was diluted with the same compost (without fungus) at the concentration 1 : 100 (v : v). This substrate was used to make small blocks (4 x 4 x 4 cm^3) with a mechanical apparatus (F.A.O. patent). Each block received a tomato seedling which was cultured during 3 weeks in a glasshouse. The blocks with the seedlings were then transferred to the plots (2.5 x 2.5 m ; 25 plants per plot, 0.5 apart) separated from one another by 2 m. The treatments were arranged in a randomised complete block design with 10 replicates. The control treatment consisted of blocks without fungus. This experiment was conducted from May to July (maximal temperature < 35°C) on the same soil as that used in the section 1/. The height, the mortality, the shoot and root biomass were determined after two month culture. Every month, from transplanting to the end of the experiment, one tomato plant was uprooted from each plot. The nematodes were extracted from the roots as described above. A 250 g sample of the soil surrounding the plant was sampled in each plot and the nematodes were extracted (Seinhorst, 1962). The data were treated with a one way analysis of variance and the mean values were compared with the Student's t test ($P < 0.05$). For nematodes, data were previously transformed by log(x+1).

RESULTS

The juveniles of M. mayaguensis were trapped by all the fungal strains (Table 2). The higher rates were recorded with ORS 18692 S7, S 30, S 31 and S 42. The higher trapping activities against M. incognita were observed with S 30, S 31 and S 42. On the opposite, M. javanica juveniles were only affected by the fungal strain S 31.

The growth of tomato plants was significantly increased when the strains ORS 18690 S2, ORS 18692 S7 and S42 have been inoculated (Table 3). The fungal isolate S31 has only stimulated the root development. The numbers of juveniles of M. mayaguensis per plant were significantly lower in the fungal treatments than in the control. The same effect was observed with the gall indexes excepted with ORS 18692 S7 (Table 3).

The fungal growth was increased by 7 bacterial isolates (S22, S51, S73, G10, G33, G36 and SG9 when bacteria were testing for direct trophic effect (liquid contac)(Table 4). Only 3 bacterial isolates have improved the fungal growth when the microorganisms were confrontated by a gaseous way. Seven bacterial isolates (S51, S109, G36, G93, G95, SG8 and SG9) enhanced the predatory activity of the fungus T 41 on M. mayaguensis (Table 4).

Table 2. Predatory activity (expressed as % of trapped Meloidogyne spp. juveniles) of the fungal isolates against 3 species of Meloidogyne in axenic conditions

Fungal isolates	M. mayaguensis	M. incognita	M. javanica
ORS 18690 S2	11 c (1)	0	0
ORS 18692 S5	26 b	3 b	0
ORS 18692 S7	74 a	0	0
S 30	78 a	65 a	0
S 31	82 a	70 a	20
S 42	82 a	60 a	0
BF 10	10 c	4 b	0
BF 74	9 c	2 b	0
BF 80	14 c	2 b	0
SOSU 2	8 c	16 b	0

(1) data in the same column followed by the same letter did not significantly differ according to the one way anlysis of variance ($P < 0.05$)

Table 3. Effect of the fungal isolates on the growth of tomato plants infested with 100 juveniles of M. mayaguensis per plant and on the development of the nematode

Fungal strains	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Number of gallsper plant	Number of juveniles per plant
Control	23.1 b [1]	101 b	21.3 a	7046 a
ORS 18690 S2	31.3 a	194 a	15.4 b	4008 b
ORS 18692 S7	31.3 a	184 a	18.3 ab	2112 b
S 31	22.3 b	174 a	13.6 b	3611 b
S 42	31.9 a	163 a	14.4 b	2470 b
BF 10	21.7 b	109 b	12.7 b	2527 b
SOSU 2	22.9 b	140 ab	12.4 b	2132 b

[1] data in the same column followed by the same letter did not significantly differ according to the one way analysis of variance ($P < 0.05$)

Table 4. In vitro effect of fluorescent Pseudomonas on the radial growth (direct and gazeous confrontation) and predacious activity of Arthrobotrys sp. T41. *: significantly different from the control according to Student's t-test ($P<0.05$). **: significantly different from the control according to the Student's t-test ($P<0.01$)

Bacterial treatments	Radial growth (mm) Direct confrontation	Radial growth (mm) Gazeous confrontation	Predacious activity (%) (trapped J2s/total J2s)
Control	63,5	13,8	45,2
S22	69,9 **	16,8	56,9
S51	69,2 **	15,5	60,0 *
S73	67,0 *	19,0 **	55,4
S109	65,8	16,5	78,6 *
G10	66,2 *	16,1	39,4
G12	63,8	16,3	46,9
G33	67,0 **	18,0 *	37,4
G36	69,4 **	17,9 *	59,0 *
G93	65,1	16,0	75,0 *
G95	61,9	17,3	61,3 *
SG1	65,4	17,4	58,3
SG8	66,2	17,3	72,9 *
SG9	66,5 *	17,1	81,7 *
SG12	63,8	16,3	37,3
SG18	62,7	14,1	35,4
SG19	61,9	14,1	41,9

The two main plant-parasitic nematodes genus identified in the tobacco root systems and in the soil were Meloidogyne spp. and Rotylenchulus reniformis (Table 5). No effect of the fungus A. oligospora T41 on Meloidogyne has been recorded (Table 5). Only 2 bacterial isolates (G93 and SG19), inoculated without T41, have significantly inhibited the multiplication of the root-knot nematodes. However, when the dual inoculation (T41 + bacterial strain) was performed, the number of juveniles per plant has been significantly decreased in the treatments with G10, G36 and G95. The multiplication of R. reniformis has been inhibited in the bacterial treatment S73 (Table 5). When the fungus was added, an inhibition of the nematode development has been recorded in all the treatments (Table 5) excepted in the treatment SG18 + T41.

At the end of the field experiment (2 months), the fungal treatment was responsible for a significant increase in the height, shoot and root biomasses of the tomato plants and a significant decrease of the mortality (Table 6). Moreover the number of juveniles of Meloidogyne per gram of root biomass and per dm^3 of soil was significantly greater in the control than in the treatment with ORS 18692 S7 (Fig. 1).

Table 5. Effect of the bacterial strains on the number of juveniles of Meloidogyne spp. and Rotylenchulus reniformis per tocacco plants inoculated or not with the nematophagous fungus Arthrobotrys sp. T41. * : significantly different from the control (not inoculated treatment) according to the Student's t test ($P < 0.05$). ND : Not Determined.

Bacterial strains	Meloidogyne spp.		Rotylenchulus reniformis	
	Bacteria alone	Bacteria + T41	Bacteria alone	Bacteria + T41
Control(without bacteria)	98944	90080	79452	41950 *
S22	186820	75890	54200 *	20780 *
S51	33410	35650	118240	ND
S73	23674	194411	35790 *	43270 *
S109	71720	104180	70040	29840 *
G10	67010	23000 *	75280	31320 *
G12	48343	49655	98630	21350 *
G33	57660	74562	65700	41137 *
G36	75370	12740 *	80790	43530 *
G93	14806 *	84212	43526 *	39925 *
G95	68740	7250 *	86131	47310 *
SG1	63700	89990	73160	26010 *
SG8	147072	101551	59050 *	29670 *
SG9	59640	146770	43730 *	28670 *
SG12	48138	57910	69826	37400 *
SG18	35140	50140	51890 *	75160
SG19	7640 *	103643	42710 *	44070 *

Table 6. Effect of Arthrobotrys oligospora ORS 18692 S7 on height, mortality, average shoot and root biomass of tomato plants in the field experiment after 2 month culture. [(1)] : for each parameter, data in the same lign followed by the same letter did not significantly differ according the one way analysis of variance ($P < 0.05$).

Parameters	Compost blocks without fungus	Compost blocksWith fungus
Height (cm)[(1)]	23.2 b	31.8 a
Mortality (%)	36.8 a	16.4 b
Shoot biomass (g dry weight per plant)	7.1 b	12.6 a
Root biomass (g dry weight per plant)	0.7 b	1.8 a

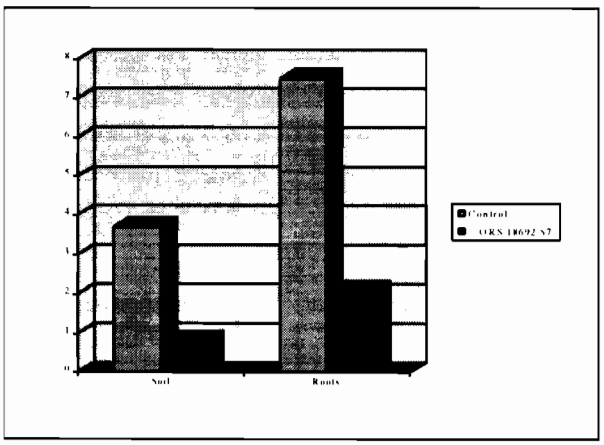

Fig. 1. Effects of the nematophagous fungus ORS 18692 S7 on the multiplication of Meloidogyne spp. juveniles in tomato roots and in the soil. For each parameter (root and soil), the columns indexed with the same letter are not significantly different ($P < 0.05$). The data of the nematode population in the soil are expressed as x 100, in the roots, the numbers are calculated per g of dry weight of root biomass and divided by 10^5.

DISCUSSION

The different fungal isolates of Arthrobotrys from Senegal and Burkina Faso were able to trap the juveniles of M. mayaguensis and M. incognita. All these fungi could be good candidates for biological control in coffe plantations as M. incognita has occured for many years in separate or mixed populations (with M. exigua, M. coffeicola). Moreover the fungal isolates can act against M. mayaguensis. This very damaging Meloidogyne species has spread throughout

vegetable-producing areas in West Africa and is now present under various agro-climatic conditions (Mateille et al., 1994).

In the glasshouse experiment, the fungi had a great influence on the growth of tomato plants in spite of a low rate of inoculation (1 : 100, v :v). Compared to the concentrations commonly used in the controlled ectomycorrhization (1 : 10, v :v)(Duponnois & Garbaye, 1991), these results suggest that the nematophagous fungi have a great capacity to colonize the soil and consequently trapped the juveniles before infecting the roots. The decrease of the number of galls per plant in the fungal treatments shows this physiological advantage. Moreover these fungi can also use phytoparasitic nematodes as an energy source and it can be assessed that more larger is the nematode population, more important will be the fungal development.

However all these experiments have been performed in controlled conditions and during a short period (about one month). It is well known that the efficacy of these fungi strongly interact with environmental factors such moisture, pH, temperature and organic matter (Mosse, 1972). Recent researches have shown that the biological activities of soil microorganisms can be enhanced by some rhizosphere bacteria such as fluorescent Pseudomonas (Duponnois et al., 1993 ; Mateille & Duponnois, 1996). The results presented in this paper focussed on the great interactions between this group of bacteria and the nematophagous fungus. These bacterial isolates have been called NHB (Nematophagous Helper Bacteria)(Duponnois et al., 1998). From a practical point of view, it is generally assessed that the production of spores and mycelial biomass by nematophagous fungi is very limited (Cayrol, 1988). Moreover, the use of NHB can decrease the quantities of fungal inoculum added to the soil because of their benefical effect on the saprophytic growth and the predacious activities of the fungal strains. These NHB can also enlarge the effect of this practice on other pathogenic microorganisms such as Rotylenchulus reniformis which is also a widespread plant parasitic nematodes. As we have demonstrated that the compost blocks can be a good cultural support for the biological control against nematodes with nematophagous fungi, it is easly possible to add together the fungus and a selected bacterial strain inside the block. This new concept of the biological control could integrated the properties of the fungus and those from the bacteria (Plant Growth Promoting Rhizobacteria, etc) and their beneficial interactions. However, the mechanisms involved between these two types of microorganisms must be elucidated in order to increase the efficiency of this dual inoculation.

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