Chapter 24

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

R. Duponnois[1], J.L. Chotte[1], A.M. Bâ[2], S. Roussos[3]

[1] *Institut de Recherche pour le Développement (IRD), Laboratoire de Biopédologie. BP 1386, Dakar. Sénégal;* [2] *Institut Sénégalais de Recherche Agronomique (ISRA), BP 1386, Dakar. Sénégal,* [3] *IRD. Laboratoire de Mycologie et des FMS (LAMY-FMS). IFR-BAEM; Universités de Provence et de la Méditerranée, ESIL, Case 925; 163 Avenue de Luminy. F-13288, Marseille Cedex 9 France*

Running title: Helper bacteria of nematophagous fungi

1. Introduction

Many genera and species of plant parasitic nematodes are associated with coffee. These cause great financial losses to the coffee farmers. Among these pathogens, root-knot nematodes (*Meloidogyne* sp.) 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 ageing 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 were 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 impractical 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 nematode population in the soil.

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The control practices used by many farmers are 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. However, they are expensive and toxic when used improperly. Thus, other control techniques such as biological control have been investigated. Besides various micro-organisms tested against *Meloidogyne* sp. such as arbuscular mycorrhizae (Hussey and Roncadori, 1982), eggs parasitic fungi (*Verticillium chlamydosporium*) (Kerry, 1990; Bourne *et al.*, 1994), rhizobacteria (Racke and 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* sp. was based on the use of tests performed in axenic or controlled conditions (*in vitro* tests or glasshouse experiments with disinfected substrates). When these micro-organisms 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 reply from interactions with the soil microbial community in the soil. In the laboratory of Biopedology (IRD, Dakar), we have developed a scientific programme based on:

(i) the (i) screening of efficient fungal strains for trapping juveniles of *Meloidogyne* sp.,
(ii) determination of potential effects of rhizobacteria on *in vitro* growth and nematode-trapping activity of *Arthrobotrys oligospora*, population development of plant parasitic nematodes
(iii) to develop a control method with the fungal strain incorporated into compost blocks. Tobacco and tomato plants, very susceptible to *Meloidogyne* sp., were used in these experiments.

2. Experimental

2.1. TEST OF THE POTENTIAL OF NEMATOPHAGOUS FUNGI AGAINST *MELOIDOGYNE* SP.

A Collection of nematophagous fungi isolated from several vegetable-producing areas in Senegal and Burkina Faso was maintained aseptically in 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* sp. *(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 $g.l^{-1}$).

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	Duponnois R.
Arthrobotrys oligospora	ORS 18692 S5	Senegal	Duponnois R.
Arthrobotrys oligospora	ORS 18692 S7	Senegal	Duponnois R.

[(1)] INERA, Protection des Végétaux, BP 403, Bobo Dioulasso, Burkina Faso

One week later, 100 second-stage juveniles suspended in a water droplet were placed on these fungal cultures. After two days, the numbers of juveniles trapped by the fungus were counted under a dissecting microscope. Each combination *Meloidogyne* sp.-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* sp. 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 samplings from each fungal culture on Petri dishes were put into flasks. The cultures were incubated for five 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 distributed 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 one 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 between 20-35°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 1-2 cm pieces and placed in a mist chamber for two weeks to recover hatched juveniles (Seinhorst, 1950). Roots were then oven-dried and weighed. The data were treated with 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).

2.2. STUDY OF THE POTENTIAL EFFECTS OF RHIZOBACTERIA ON THE ANTAGONISTIC ACTIVITY OF *ARTHROBOTRYS OLIGOSPORA* AGAINST *MELOIDOGYNE* SP.

Study of the potential effects of rhizobacteria on the antagonistic activity of *Arthrobotrys oligospora* against *Meloidogyne* sp. was carried out as described by Duponnois and Bâ (1998). The authors have demonstrated that a large nematophagous fungus population was associated with the presence of bacteria belonging to the group of fluorescent *Pseudomonas*. Attempts were made 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* sp. 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 sulphate solution ($MgSO_4$, 0.1 M). The control treatment was prepared in the same way from a Petri dish containing the same agar medium but 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 and Garbaye, 1990). Another set of experiment was made 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 by the bacterial strains (uninoculated for control treatment). Since the wall separating the two compartments did not touch the lid of the dish, the gas diffusion from one side to the other was permitted.

In both the experiments, two dishes, each with three mycelial plugs, were prepared for each treatment and 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 directions 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 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 *M. mayaguensis* suspended in 100 µl sterile distilled water were placed on the fungal cultures. Populations of *M. mayaguensis* were reared on tomato (*L. 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 collect the juveniles (J2) (Seinhorst, 1950). After 48 hours, the numbers of the juveniles trapped by the fungus were counted. The data were statistically treated as described above.

Tobacco seedlings (*Nicotiana tabacum* L. var. Paraguay x Claro) were grown in 60 ml polythene pots filled with an autoclaved sandy soil (140°C, 40 min) containing (%) clay 3.9, silt 2.9, sand 92.2, carbon 3.7, nitrogen 0.45 (pH-$H_2$0 8.3) and 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 was grown in one litre flask filled with 0.5 litre of 0.3% TSB medium for two weeks at 25°C. The fungal suspension was then filtered and the mycelium was collected, washed three times in $MgSO_4$ 0.1-M solution and finally re-suspended in $MgSO_4$ 0.1 M solution. The bacterial isolates were cultured in 3 $g.l^{-1}$ liquid Difco tryptic soy broth in glass flasks under agitation for eight days at 25°C, centrifuged (2400 g, 10 min) and the pellet was re-suspended in $MgSO_4$ 0.1 M solution. The control treatments were performed by injecting 1-ml of $MgSO_4$ 0.1 M solution without either fungus or bacteria in the soil. There were ten replicates per treatment. After two-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 (temperature between 20-35°C, about 15 hours light per day). After two-month culture, the plants were harvested and the roots were 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.

2. 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 described above. 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 three 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) 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 2.1. The height, mortality and shoot and root biomass were determined after two months. 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).

3. Results and Discussion

The juveniles of *M. mayaguensis* were trapped by all the fungal strains (Table 2).

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

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

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 were inoculated. The fungal isolate S31 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).

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 galls per 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

*: 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 ($P<0.01$).

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

The fungal growth was increased by seven bacterial isolates (S22, S51, S73, G10, G33, G36 and SG9 when bacteria were tested for direct tropic effect (liquid contact) (Table 4). Only three bacterial isolates improved the fungal growth when the micro-organisms confronted by 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).

The two main plant-parasitic nematodes genus identified in the tobacco root systems and in the soil were *Meloidogyne* sp. and *Rotylenchulus reniformis* (Table 5). *A. oligospora* T41 did not affect *Meloidogyne* sp. Only two bacterial isolates (G93 and SG19), inoculated without T41, 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 decreased significantly in the treatments with G10, G36 and G95. The multiplication of *R. reniformis* was inhibited in the bacterial treatment S73 (Table 5). When the fungus was added, an inhibition of the nematode development was recorded in all the treatments, with the exception of treatment SG18 + T41.

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 treatment	Direct confrontation Radial growth (mm)	Gazeous confrontation Radial Growth (mm)	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

*: significantly different from the control according to Student's test (P<0.05).
** : significantly different from the control according to Student's test (P<0.01).

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.

Strains of	*Meloidogyne* spp.		*Rotylenchulus reniformis*	
Bacteria	Bacteria alone	Bacteria + T41	Bacteria alone	Bacteria + T41
Controls[1]	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 *

[1] Without bacteria
* : significantly different from the control (not inoculated treatment) according to the Student's t test ($P < 0.05$). ND : Not Determined.

At the end of the field experiment (two months), the fungal treatment was responsible for a significant increase in the height, shoot and root biomass of the tomato plants and a significant decrease of the mortality (Table 6).

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.

Parameters	Compost block without fungus	Compost blocks with fungus
Height (cm)*	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 eight per plant)	0,7 b	1,8 a

*: for each parameter, data in the same lign followed by the same letter did not significantly differ according to the one way analysis of variance (P<0.05)

Moreover the number of juveniles of *Meloidogyne* sp. 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).

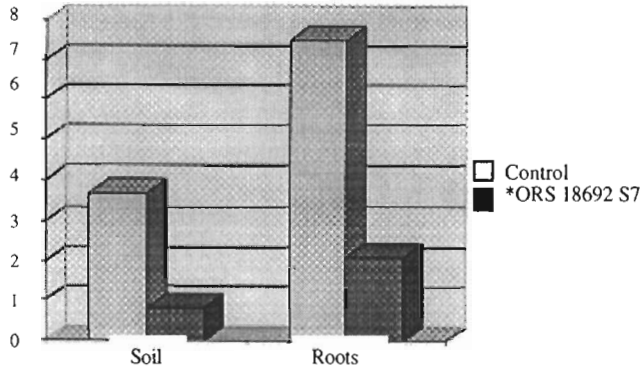

Figure 1. Effects of the nematophagous fungus ORS 18692 S7 on the multiplication of *Meloidogyne* spp. juveniles in tomato roots and in the soil. For each parameter (root and soil), the columns indexed with the same letter are not significantly different (P < 0.05). The data of the nematode population 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.

The different fungal isolates of *Arthrobotrys* from Senegal and Burkina Faso were able to trap the juveniles of *M. mayaguensis* and *M. incognita*. Thus, these could be good candidates for biological control in coffee plantations as *M. incognita* has occurred for many years in separate or mixed populations with *M. exigua, M. coffeicola*. Moreover, the fungal isolates can act against *M. mayaguensis*, which has caused serious damages and has spread throughout vegetable-producing areas in West Africa. It is now present under various agro-climatic conditions (Mateille *et al.*, 1996).

In the glasshouse experiment, the fungi showed 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 and Garbaye, 1991), these results suggested 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 showed this physiological advantage. Moreover, these fungi can also use phytoparasitic nematodes as an energy source.

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 micro-organisms can be enhanced by some rhizosphere bacteria such as fluorescent *Pseudomonas* (Duponnois *et al.*, 1993; Mateille and Duponnois, 1996). Present findings focus on the great interactions between this group of bacteria and the nematophagous fungus. These bacterial isolates have been termed as Nematophagous Helper Bacteria (NHB) (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). Use of NHB can decrease the quantities of fungal inoculum added to the soil because of their beneficial effect on the saprophytic growth and the predacious activities of the fungal strains. These NHB could also enlarge the effect of this practice on other pathogenic micro-organisms such as *Rotylenchulus reniformis*, which are also widespread plant parasitic nematodes. As we have demonstrated the compost blocks could be a good culture support for the biological control against nematodes with nematophagous fungi. It is simple to add together the fungus and a selected bacterial strain inside the block. This new concept of the biological control could integrate 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 micro-organisms needs to be elucidated in order to increase the efficiency of this dual inoculation.

4. Summary

Present studies on the biological control with nematophagous fungi against the root-knot nematodes showed that the fungi could 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 culture method was developed using compost blocks in which the fungus was inoculated. In the field experiment, the growth of tomato plants increased while the nematode development decreased. However, all these experiments have been performed during a short period. It was found that the fungus could be associated with bacterial isolates, called NHB, which stimulated the fungal activity against *Meloidogyne*. This, however, also involved a depressive effect on other plant parasitic nematodes such as *R. reniformis*.

5. Reference

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