RESEARCH/INVESTIGACIÓN

ROOT ENDOPHYTIC STATUS OF WEST AFRICAN BIOCONTROL AGENTS AND IMPLICATIONS FOR ROOT-KNOT NEMATODE MANAGEMENT

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ABSTRACT

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The potential of four fungal biocontrol isolates (*Trichoderma asperellum* T-12, *Pochonia chlamydosporia* Pc-1, *Kuklospora kentinensis* BEN302, *Aspergillus allahabadii* BEN03) from West Africa to endophytically colonize tomato plants and provide *in planta* protection against root-knot nematodes (*Meloidogyne* spp.), was assessed in pots in the greenhouse. Colonization assessment at 11 wk after inoculation indicated that all the fungal isolates were able to internally develop and colonize tomato roots. The root dip treatments with the fungi led to significant inhibition of root-knot nematode reproduction, juvenile hatching, and root galling damage. Pre-planting treatment with *T. asperellum* T-12 showed the greatest nematode control, reducing root galling severity and nematode reproduction by 47% and 67%, respectively, and significantly inhibiting egg hatch by up to 85%, compared to the control. Endophytic protection by the individually inoculated fungal isolates also led to significantly improved tomato growth of shoots and roots. Such *in planta* host plant nematode suppression lends itself to novel, cost-effective, and environmentally suitable pest management options that can be efficiently integrated into seedling production systems. This study provides strong evidence and justification for exploring other mechanisms such as systemic effects of the fungi and, in particular, the effect of fungi on reducing egg hatching.

Key words: Benin, biological control, *Meloidogyne* spp., nematophagous fungi, vegetables

RESUMEN

Affokpon, A., A. C. Djihinto, E. N. D. Coffi, D. L. Coyne, y J. Coosemans. 2018. Estado endofítico de la raíz de los agentes de biocontrol del África occidental e implicaciones para el manejo del nematodo agallador. Nematropica 48:92-100.

El potencial de cuatro aislamientos del hongo de control biológico (*Trichoderma asperellum* T-12, *Pochonia chlamydosporia* Pc-1, *Kuklospora kentinensis* BEN302, *Aspergillus allahabadii* BEN03) de África occidental, para colonizar endofiticamente plantas de tomate y proporcionar protección *in planta* contra nematodos agalladores (*Meloidogyne* spp.), se evaluó en macetas en el invernadero. La evaluación de la colonización a las once semanas después de la inoculación indicó que todos los aislados fúngicos pudieron desarrollar y colonizar internamente las raíces del tomate. Los tratamientos de inmersión de las raíces con los hongos condujeron a una inhibición significativa de la reproducción del nematodo agallador, la eclosión juvenil, y el daño de la irritación de la raíz. El tratamiento previo a la siembra con *T. asperellum* T-12 mostró el mayor control de nematodos, reduciendo la severidad de irritación de las raíces y la reproducción de nematodos en un 47% y 67%, respectivamente, e inhibiendo significativamente la eclosión

hasta en un 85%, en comparación con el control. La protección endofítica por los aislados de hongos inoculados individualmente también condujo a un crecimiento significativo de los brotes y raíces del tomate. Tal supresión de nematodos por hospedadoras *in planta* se presta a opciones de manejo de plagas nuevas, rentables y ambientalmente adecuadas que pueden integrarse de manera eficiente en los sistemas de producción de plántulas. Este estudio proporciona una fuerte evidencia y justificación para explorar otros mecanismos, como los efectos sistémicos de los hongos y, en particular, el efecto de los hongos en la reducción de la eclosión de los huevos.

Palabras claves: Benin, control biológico, hongos nematófagos, *Meloidogyne* spp., vegetales

INTRODUCTION

In African farming systems, root-knot nematodes (*Meloidogyne* spp.) are the greatest single biotic threat to agricultural productivity throughout the continent (Coyne *et al*., 2009), and in urban and peri-urban vegetable systems in West Africa as a key reason for the overuse, misuse, and abuse of synthetic pesticides (James *et al*., 2010).

Recently, a number of studies have investigated the presence and evaluated the suitability of fungal isolates for providing the biocontrol of nematode pests in West Africa with *Trichoderma* spp., arbuscular mycorrhizal fungi, *Pochonia chlamydosporia* and *Aspergillus allahabadii*, demonstrating promising potential against root-knot nematodes in pots and field experiments (Tchabi *et al*., 2007, 2016; Affokpon *et al*., 2010, 2011a, 2011b, 2015). Furthermore, their application in double-cropping systems in the field has provided strong root-knot nematode suppression, leading to significant yield increases and demonstrating field persistence over two consecutive crop cycles (Affokpon *et al*., 2011a, 2011b). During these preliminary studies, biocontrol agents were applied in the field by broadcasting, which could raise the costs of application and reduce profit margins on crop returns (Sikora and Pocasangre, 2006). Consequently, the economical aspect has stimulated interest towards improving the efficacy and efficiency of these fungal biocontrol agents. One such avenue was to assess the ability of the fungi to colonize the target host crop as root endophytes, and further to provide nematode management. The potential of root endophytic fungi to reduce root-knot nematode infestation was first reported from arbuscular mycorrhizal fungi in vegetable transplants (Sikora and Schonbeck, 1975). This report indicated reduced plant infection by root-knot nematodes, following pre-transplant inoculation of tomato seedlings with arbuscular mycorrhizal fungi. Similar biocontrol effects were reported with non-pathogenic isolates of *Fusarium oxysporum*, which reduced the rotting of excised banana roots caused by *Pratylenchus goodeyi* (Speijer, 1993), and with *F. oxysporum* isolate FO162, which reduced the number of *M. incognita* that penetrated the roots (Dababat and Sikora, 2007).

The current study was undertaken to establish if beneficial microorganisms (Affokpon *et al*., 2010, 2011a, 2011b, 2015) could endophytically protect against root-knot nematodes, and to develop economical mechanisms for delivering and implementing suitable application systems to optimize the effectiveness of beneficial microorganism against root-knot nematodes.

MATERIAL AND METHODS

Biocontrol agents and inoculum preparation

Trichoderma asperellum T-12, *Pochonia chlamydosporia* Pc-1, *Kuklospora kentinensis* BEN302, and *Aspergillus allahabadii* BEN03 native to Benin, were used for the greenhouse pot experiments. Isolates were selected based on their rhizosphere competency and biological potential against root-knot nematodes (Affokpon *et al*., 2010, 2011a, 2011b, 2015). The isolates of *Trichoderma* and *Aspergillus* were cultured on potato dextrose agar (Affokpon *et al*., 2010), whereas *P. chlamydosporia* and the arbuscular mycorrhizal fungus *K. kentinensis* BEN302 were sub-cultured on sand maize medium (Affokpon *et al*., 2015) and sorghum (Affokpon *et al*., 2011b), respectively. Suspensions of the fungi were prepared and spore or chlamydospore population densities determined according to each isolate (Affokpon *et al*., 2010, 2011a, 2011b, 2015). Fungal suspensions were individually adjusted to 50 cm^3 and transferred to separate plastic bowls for inoculation of seedlings. A mixture (50 cm^3) of the four fungal isolates was additionally prepared to serve as combination treatment to explore possible synergistic or antagonistic effect between fungal isolates. After 1-hr root dipping, initial inocula per plant, according to the treatment, were 2.67 x 10^6 chlamydospores for *P. chlamydosporia* Pc-1, 1.28 x 10^9 spores of *T. asperellum* T-12, 4.79 x 10^9 spores of *A. allahabadii* BEN03 and 459 spores for arbuscular mycorrhizal fungi. In the combination treatment, the initial inoculum per individual fungi

was not determined, but total spores were 1.25 x 10^9 per plant.

Experimental details

The study was conducted in the greenhouse at the IITA research Station, Cotonou (06°25 N, 002°19 E, 18 m asl). The experiment included the four fungal isolates used separately, the combination of all isolates and two non-fungal-amended controls, a nematode positive control, and a nematode-free control.

Tomato seeds (cv. Tropimech) were sown singly into seed trays containing sterilized chopped coconut husk, and maintained in the greenhouse for 3 wk at ambient temperature of 29 ± 5°C. Seedlings (five per treatment) were then gently removed from the tray, and the roots carefully washed free of seedling medium with sterile distilled water (SDW), and then suspended in a plastic bowl containing the fungal suspensions for 1 hr. Thereafter, the seedlings were singly transferred to 2-L plastic pots containing 1,000 cm³ of non-sterilized soil (loam sandy, organic matter: 1.34%, C/N: 10.7, pH: 6). Prior to use, two samples of 250 cm³ each were removed from the bulk soil and the initial root-knot nematode density (Pi) determined after nematode extraction using centrifugation techniques (Affokpon *et al*., 2011a). The composite soil had a Pi of 300 root-knot nematode second stage juveniles (J2) per 250 cm³ soil, corresponding to 1,200 J2 per pot.

Pots were arranged in the greenhouse in a completely randomized design with five replicate pots per treatment. Plants were watered daily using rain water and maintained for 11 wk before harvest. The pots received fertilizer once with 25 cm³ of urea (46% N) solution after dilution to 2% using tap water, at 6 wk after transplanting.

Estimation of the final densities of the fungi

The final fungal densities in the soil and roots in control pots and pots treated with *T. asperellum* T-12, *P. chlamydosporia* Pc-1, and *A. allahabadii* BEN03 were estimated at harvest using the soil dilution plating technique (Kerry and Bourne, 2002). To determine soil fungal densities, four soil cores per pot were removed using a 2-cm-diam. cork borer. All cores from the same pot were combined, and a 2 cm³ sub-sample added to 18 cm³ of 0.05% sterile agar solution. A dilution series, 10^{-1} to 10^{-3}, was prepared from the soil suspension and 0.2 cm³ of each dilution was plated onto three 9-cm Petri dishes containing the semi-selective medium recommended for each fungus and incubated at appropriate temperature (Affokpon *et al*., 2015). Root colonization, root total CFU (internal and surface), and internal root CFU were assessed at harvest. The procedure used to assess root total fungal growth was similar to that described by Affokpon *et al*. (2011a) using 1-g root subsample per pot. However, to evaluate internal root colonization growth, a 1-g representative root sub-sample from chopped roots of each pot was surface-sterilized with 1% NaOCl for 3 min, rinsed 5 times in sterile distilled water before being crushed in 0.05% water agar with ethanol-sterilized pestle and mortar.

For the arbuscular mycorrhizal fungus treatment, root colonization was assessed using 10, 2-cm root sections per pot after clearing in 10% KOH and staining with 0.05% trypan blue in lactic acid/glycerol (Koske and Gemma, 1989). The frequency and intensity of root mycorrhization were then estimated (Affokpon *et al*., 2011b).

Effect of fungal application method on plant growth

The effect of root dip treatments with different fungi on tomato growth was assessed at the end of the experiment. At harvest shoot length and shoot and root fresh weights were recorded.

Estimation of final nematode densities and root galling

Final nematode densities were assessed from soil and roots. Soil J2 were extracted from 250 cm³ soil sub-samples per pot and nematode root densities from 5 g root sub-samples by the centrifugation technique. Nematode reproductive factor (RF) was then calculated as the ratio Pf/Pi, with Pf the final nematode densities per pot, comprising soil and root J2 and eggs per pot, and Pi the initial nematode inoculum per pot (Affokpon *et al*., 2011a).

Prior to root nematode extraction or fungal isolation, the whole root system per pot was assessed for galling index on a scale of 0-10 with 0 = no knots on roots and 10 = all roots severely knotted or no root system (Bridge and Page, 1980).

Effect on juvenile hatching

The ability of each isolate to colonize eggs of root-knot nematodes was also estimated. Five egg masses per root system were removed, rinsed five times with SDW, and crushed using glass rods in a dish to release eggs. Eggs were then separated from debris and hatched J2 by filtering through a 200-µm pore sieve and the number of eggs counted. Egg suspensions, adjusted to 20 cm³ containing at least 400 eggs per treatment unit (pot), were transferred to Petri dishes and maintained in the laboratory temperature for 7 days. Thereafter, the number of unhatched J2 per Petri dish was counted under

compound microscope (Olympus CX31) and the percentage of hatched J2 estimated.

Statistical analysis

Before analyzing, data on nematode densities and fungal counts were transformed to log(x + 1) and percentage of root mycorrhization and juvenile hatching to $\arcsin(x/100)^{1/2}$, so that data conformed to normal distribution. All data were subjected to ANOVA using Statistica package (StatSoft, 2001). When the univariate *F*-test was significant for a parameter, means were separated using Fisher's LSD test. Differences referred to in the test are significant at $P \leq 0.05$.

RESULTS

Final densities of the fungi

The biocontrol agents *T. asperellum* T-12, *P. chlamydosporia* Pc-1, and *A. allahadahii* BEN03 were all re-isolated from root and soil samples from both pots inoculated individually or in full combination at 11 wk after transplanting (Table 1). Fungal colonies were also observed from surface-sterilized root suspensions for all fungal treatments. The abundance of the fungi was significantly higher ($P< 0.0001$ in all treatments) in unsterilized root suspensions than in soil and in sterilized root suspensions, with soil suspensions recording the lowest fungal densities, except for pots treated with *P. chlamydosporia*.

In both the individual or combined arbuscular mycorrhizal fungi treatments, root mycorrhization was relatively high (*Table 2*). The frequencies of root colonization by the arbuscular mycorrhizal fungi isolates were equal or higher than 50% in plants treated with *K. kentinensis* BEN 302 either alone or in combination. Root colonization intensities were also high, whether the isolates were applied alone or in combination with other fungi.

Plant growth

At 11 wk after transplanting, the root dipping application of the fungi positively affected ($P< 0.0001$) shoot and root fresh weights over the nematode positive control, but not shoot length ($F = 2.129$; $P = 0.0813$) (Table 3). Individual applications of the fungi led to significantly higher shoot ($F = 33.498$; $P< 0.0001$) and root ($F = 62.005$; $P< 0.0001$) fresh weights, when compared with the nematode positive control (Table 3). Plants treated with the combined fungal suspension had, in most cases, significantly lower shoot and root fresh weights than plants treated with fungi individually, but similar to those from the nematode positive control. Furthermore, shoot and root fresh weights were significantly higher in nematode-free soil than in all other treatments, by 98% and 93%, respectively, over the nematode positive control.

Final nematode densities and root galling

Root treatment with fungi alone or in combination resulted in significant suppression of egg production ($F = 31.4$; $P< 0.0001$) and root J2 numbers ($F = 116$; $P< 0.0001$), compared with the nematode positive control. Root J2 and eggs were reduced in all fungal treatments by up to 55% and 88%, respectively, over the nematode positive control (Table 4). Soil J2 numbers were also significantly affected ($F = 6.7$; $P = 0.0005$) by root

Table 1. Final fungal densities from tomato (cv. Tropimech) root (CFU[t] x 10^4 per g^{-1} root) and rhizosphere (CFU x 10^4 per cm^3 soil) 11 weeks after transplanting in unsterilized soil naturally infested with 1,200 juveniles of *Meloidogyne* sp. in 1,000 cm^3 pots.

Substrates	*Pochonia chlamydosporia* Pc-1		*Trichoderma asperellum* T-12		*Aspergillus allahabadii* BEN03	
	Single[u]	Combination[v]	Single	Combination	Single	Combination
Unsterilized root[w]	11.2 a[x]	260 a	123 a	192 a	107 a	Nd[y]
Surface sterilized root[z]	1.0 c	2.3 c	70 b	53 b	30 b	nd
Rhizosphere soil	3.2 b	8.8 b	9.3 c	6.8 c	2.6 c	nd

[t]CFU: Colony forming units.
[u]Single: isolate applied alone.
[v]Combination: mixture of different isolates.
[w]Unsterilized root: roots washed only with sterile distilled water before being used for assessing root CFU.
[x]Means in columns followed by different letters are significantly different ($P < 0.05$) based on Fisher LSD test using one-way ANOVA.
[y]nd: fungal densities were not determined due to the overgrowth of *A. allahabadii* colonies by some fast-growing fungi.
[z]Surface sterilized root: roots were surface-sterilized with 1% NaOCl for 3 min.

Table 2. Tomato (cv. Tropimech) root colonization by arbuscular mycorrhizal fungi (AMF) in unsterilized soil 11 weeks after transplanting in unsterilized soil naturally infested with 1,200 juveniles of *Melodogyne* sp.in 1,000 cm³ pots.

AMF treatments[z]	Frequency (%)	Intensity (%)
Kuklospora kentinensis BEN302 (single)	80 (70-90)	54 (41-66)
K. kentinensis BEN302 (combination)	50 (30-70)	42 (30-50)

[z]Single: isolate applied alone, Combination: mixture of different isolates.

Table 3. Mean shoot length, shoot fresh weight, and root fresh weight of tomato (cv. Tropimech) 11 weeks after transplanting in unsterilized soil naturally infested with 1,200 juveniles of *Melodogyne* sp. in 1,000 cm³ pots.

Treatments	Shoot length (cm)	Shoot fresh weight (g)	Root fresh weight (g)
Trichoderma asperellum T-12	37.6 a[z]	30.5 c	13.7 c
Aspergillus allahabadii BEN03	43.8 a	38.1 b	15.7 b
Pochonia chlamydosporia Pc-1	33.8 a	38.0 b	15.2 b
Kuklospora kentinensis BEN302	38.8 a	27.8 cd	12.1 d
Combination of fungi	40.2 a	26.3 de	10.5 e
Control (sterilized soil)	39.2 a	46.4 a	18.6 a
Positive Control (unsterilized soil)	36.0 a	23.6 e	9.7 e

[z]Means in columns followed by different letters are significantly different ($P \leq 0.05$) based on Fisher LSD test using one-way ANOVA. Combination: mixture of different isolates.

Table 4. Final root-knot nematode population densities, reproductive factor (*RF*), and gall index of tomato (cv. Tropimech) eleven weeks after transplanting in unsterilized soil naturally infested with 1,200 root-knot nematode juveniles in 1,000 cm³ pots.

Treatments	Eggs/ 5 g root	Juveniles/ 5 g root	Juveniles/250 cm³ soil	*RF* = *Pf/Pi*[y]	Gall index[x] (0-10)
Trichoderma asperellum T-12	1,886 d[z]	1,383 de	47 c	7.6 c	3.6 c
Aspergillus allahabadii BEN03	2,447 c	1,699 cd	47 c	11.0 b	5.0 b
Pochonia chlamydosporia Pc-1	2,929 b	1,184 e	55 a	10.6 b	5.6 b
Kuklospora kentinensis BEN302	2,051 d	1,897 c	52 ab	8.1 c	5.4 b
Combination of fungi	2,066 d	3,942 b	49 bc	10.6 b	5.0 b
Positive Control (unsterilized soil)	4,155 a	10,089 a	51 b	23.1 a	6.8 a
Negative Control (sterilized soil)	-	-	-	-	-

[x]Gall index was assessed on a scale of 0-10 where 0 = no galling and 10 = 91-100 % galled roots (Bridge and Page, 1980).

[y]*RF* (nematode reproductive factor) = *Pf* (final densities of eggs and juveniles per pot) / *Pi* (initial nematode inoculum per pot).

[z]Means in columns followed by different letters are significantly different ($P \leq 0.05$) based on Fisher LSD test using one-way ANOVA.

treatments, but to a varying degree compared with the nematode positive control, with plants treated with *T. asperellum* T-12 and *A. allahabadii* BEN03 having the lowest nematode population densities. Similarly, root treatments with fungi led to significant decreases ($F = 117$; $P < 0.0001$) in nematode reproduction. The biocontrol agent *T. asperellu*m T-12 inhibited nematode reproduction most, with 65% reduction over the nematode positive control. The root galling was significantly suppressed ($F = 15.410$; $P < 0.0001$) following fungal treatments (Table 4). Among fungal isolates, *T. asperellum* T-12 applied alone suppressed the root galling the best. Combining the different fungi did not reduce root galling damage further.

Juvenile hatching

The proportion of J2 hatching over 7 days was significantly lower ($F = 163.84$; $P < 0.0001$) in all fungal-treated pots than in the nematode- positive control (Fig. 1). Treatment with *T. asperellum* T-

12 led to the lowest egg hatch (15%) compared with the control.

DISCUSSION

All the fungi used in the present study either individually or in combination successfully colonized tomato rhizosphere soil and roots and persisted for at least 11wk following a 1-hr root dipping treatment. These observations indicate that high numbers of spores or chlamydospores ultimately colonized the seedling roots following this process. The application of some biological agents by root treatments, such as root dipping, have been suggested and viewed as a suitable method for reducing the amount of inoculum and overall costs compared with soil application (Spiegel and Chet, 1998; Sikora *et al*., 2000). However, most studies have focused mostly on well-known root endophytic fungi such as *Trichoderma* spp., *F. oxysporum* and arbuscular mycorrhizal fungi. In the present study, aside from *T. asperellum* T-12 and *K. kentinensis* BEN302, *P. chlamydosporia* Pc-1 and *A. allahabadii* BEN03 responded well to the dipping method, reflecting a similar study using a bare root dip treatment of tomato seedlings with *Aspergillus* species (Zareen *et al*., 2001) and *P. chlamydosporia* (Kerry and Bourne, 2002). Interestingly, the incubation of surface-sterilized root suspensions in the present study yielded fungal colony growth for all applied species. Previous reports have demonstrated that *Trichoderma* species (Harman *et al*., 2004; Bailey *et al*., 2008) and arbuscular mycorrhizal fungi (Azcón-Aguilar and Barea, 1996; Sikora *et al*., 2008) develop endophytically in the endorhiza. The current study additionally indicates clearly the *in planta* root colonization by *A. allahabadii* BEN03 and *P. chlamydosporia* Pc-1 as identified morphologically in the plates. Histological analysis of the root colonization was not undertaken in the current study to determine to what extent the internal root was colonized by the fungal hyphae. However, Lopez-Llorca *et al*., (2008) reported the colonization of root cortex cells by *P. chlamydosporia* by processing cereal roots from soil naturally infested with *Heterodera avenae*, contradicting earlier findings that the fungus does not colonize the root cortex (Bourne and Kerry, 1999). Moreover, species of *Aspergillus* have been isolated from banana Rhizome cortex collected in Thailand (Sikora *et al*., 2003). Therefore, the present study proves that *P. chlamydosporia* and *A. allahabadii* can develop endophytically within the root systems of tomato host plants.

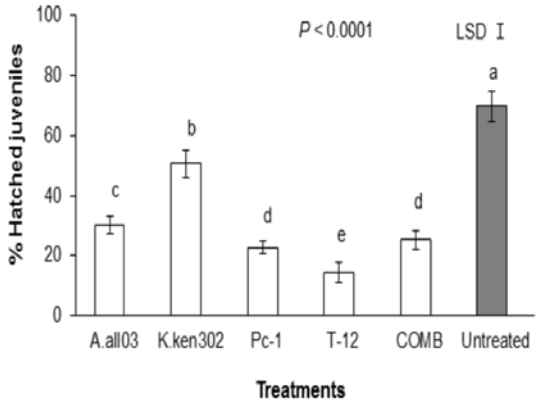

Fig. 1. Percentage of hatched juveniles of root-knot nematode eggs collected from tomato (cv. Tropimech) eleven weeks after transplanting in unsterilized soil naturally infested with 1,200 juveniles in 1,000 cm³ pots.

Pc-1: *Pochonia chlamydosporia* Pc-1, T-12: *Trichoderma asperellum* T-12, A.all03: *Aspergillus allahabadii* BEN03; K.ken302: *Kuklospora kentinensis* BEN302; COMB: mixture of different isolates; Untreated: non-fungal unsterilized positive control.

Means with different letters are significantly different after Fisher's LSD at $P \leq 0.05$ using one-way ANOVA.

Comparing plant growth in nematode-infested and nematode-free soils, the present study unsurprisingly confirms the substantial negative impact on root development by nematodes, resulting in reduction of shoot growth. The loss in plant growth due to nematodes was more important in the nematode positive control (> 90%) than the fungal treatments. These observations demonstrate that through the management of root-knot nematodes, vegetable growth and production can be substantially improved (Sikora and Fernàndez, 2005; Greco and Di Vito, 2009). In the current study, fungal root-dipping treatments of just 1 hr led to significantly reduced root-knot nematode densities at 11 wk later. Earlier studies assessing the nematicidal activity of root endophytic fungi showed that the fungi interact with the penetrating J2 and colonize their penetration sites, resulting in significant suppression of root galls and egg masses (Spiegel and Chet, 1998; Zareen *et al*., 2001; Kerry and Bourne, 2002; De La Peña *et al*., 2006; Sharon *et al*., 2007). However, those studies were conducted following inoculation using uniform single nematode species cultures under controlled conditions in sterilized soils. The present study has been conducted in unsterilized soils, originating directly from vegetable production sites in South Benin, and while dominated by *M. incognita* and *M. javanica*, harboured a mixture of root-knot nematode species (Pagan *et al*., 2015). The internal root colonization ability recorded in this study, therefore, may better reflect nematode suppression observed in fields.

In the present study, the proportion of hatched J2 in fungal treatments was lower than the control, which might indicate some biological activities of the fungi on root-knot nematode eggs. To date,

there appears to be no report that arbuscular mycorrhizal fungi inhibit J2 hatching. The reduction in J2 hatching in the arbuscular mycorrhizal fungi treatment could be due to the high mycorrhization of the roots, which disturbed physically and chemically egg mass development, resulting in higher proportion of immature eggs during the egg assessment. It may also partially result from egg infection by saprophytic fungi (e.g., *F. oxyporum*), which would explain partially the 30% of unhatched J2 in the positive nematode control.

Combinations of biocontrol agents have been recommended to cope with the often highly variable conditions common in farmers' fields and optimize the potential benefits of the various agents (Meyer and Roberts, 2002). The difference in initial fungal inoculum in the present study did not allow the comparison between the colonization levels in individual and combined applications. However, the fungi did not express any observable synergistic effect against root-knot nematodes in the combined fungal treatment. This indicates a possible antagonistic effect between some species, in agreement with Oyekanmi *et al*. (2007), causing a combination to be less effective than single isolate application, such as *T. asperellum* T-12 alone. This highlights the importance of assessment and identification of suitable combinations in biological control.

In conclusion, the present study demonstrates the suitability of the selected fungi for use in root dipping application and confirms their effect on nematode control. The *in planta* colonization of roots by these isolates possibly enables them to avoid environmental stress. The high levels of root colonization by the fungi could be substantial and especially advantageous for their use in the vegetable production sites of coastal areas, where soil leaching is a major reason for the limited success of synthetic pesticides and their overuse. Further investigations should include the interactions between the different species to identify compatible species for a synergistic biocontrol effect, in order to maximize the potential benefits of the various agents.

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