IN VITRO NEMATICIDAL EFFECT OF ENDOPHYTIC *FUSARIUM OXYSPORUM* AGAINST *RADOPHOLUS SIMILIS, PRATYLENCHUS GOODEYI* AND *HELICOTYLENCHUS MULTICINCTUS*

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ABSTRACT

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Endophytic fungi colonize most plants, causing no damage to their hosts, and often extending benefits, such as enhanced protection against various biotic and abiotic constraints. In the current study, three experiments assessed the activity of secondary metabolites of three strains of endophytic *Fusarium oxysporum* (*Emb2.4o, Eny1.31i* and *V5w2*) against the banana (*Musa* spp.) root-parasitic nematodes *Radopholus similis*, *Pratylenchus goodeyi* and *Helicotylenchus multicinctus* under different laboratory procedures. All experiments showed higher nematode mortality after 24 h exposure for each endophyte culture filtrate compared to control treatments after 24 h exposure. *Helicotylenchus multicinctus* was less sensitive to endophytic treatments than *R. similis* and *P. goodeyi*; and *R. similis* was more sensitive than *P. goodeyi* to strain *V5w2* even at lower filtrate concentrations. Based on motility of *R. similis* and *P. goodeyi*, light (14 L: 10 D h) had no significant effect on endophyte culture filtrates (*Emb2.4o* and *V5w2*). These results indicate the potential of endophytic *F. oxysporum* as an environmentally sensitive management strategy for a range of plant-parasitic nematodes on banana, although more detail is required on the identification of the toxic metabolites involved.

Key words: banana, burrowing nematode, bio-enhancement, bio-protection, biological control, endophyte, *Musa*, plant-parasitic nematode

RESUMEN

Van Dessel, P., D. Coyne, T. Dubois, D. De Waele and J. Franco. 2011. *In vitro* nematicidal effect of endophytic *Fusarium oxysporum* against *Radopholus similis, Pratylenchus goodeyi* and *Helicotylenchus multicinctus*. Nematropica 41:154-160.

Los hongos endofíticos colonizan la mayoría de las plantas sin causar daño y a veces brindan beneficios tales como la protección contra diversos agentes bióticos y abióticos. En este estudio se llevaron a cabo tres experimentos *in vitro* para evaluar la actividad de metabolitos secundarios de tres cepas de *Fusarium oxysporum* (*Emb2.4o*, *Eny1.31i* y *V5w2*) contra tres nematodos fitoparásitos del banano, *Radopholus similis, Pratylenchus goodeyi* y *Helicotylenchus multicinctus*. Todos los experimentos mostraron mayor mortalidad de los nematodos después de 24h de exposición al filtrado de cada cultivo del hongo comparados con los controles. *Helicotylenchus multicinctus* fue menos sensible a los tratameintos que *R. similis* y *P. goodeyi*; y *R. similis* fue más sensible que *P. goodeyi* a la cepa *V5w2* aún en concentraciones bajas del filtrado. Con base en la motilidad de *R. similis* y *P. goodeyi,* la luz (14 L: 10 D h) no tuvo efecto significativo sobre los filtrados del cultivo del hongo (*Emb2.4o* and *V5w2*). Estos resultados indican que *F. oxysporum* puede ser una opción de manejo ambientalmente viable para algunos nematodos fitoparásitos del banano, aunque se requiere más información acerca de los metabolitos tóxicos involucrados en el control.

Palabras clave: banano, bio-protección, control biológico, endófito, *Musa*, nematodo barrenador, nematodo fitoparásito.

INTRODUCTION

Radopholus similis, Pratylenchus goodeyi and *Helicotylenchus multicinctus* are major nematode pests affecting banana (*Musa* spp.) production across the globe (Gowen *et al*., 2005). *Radopholus similis* and *P. goodeyi* are migratory endoparasitic nematodes, while *H. multicinctus* acts both as an ectoparasitic and endoparasitic nematode, invading the superficial layers of the root cortex and causing shallow lesions (Orion and Bar-Eyal, 1995). *Radopholus similis* and *P. goodeyi* feed on root cells while migrating within the roots to new cells, causing extensive necrosis of epidermal and cortical root tissues, which leads to root death and a weakened root system (Sarah *et al*., 1996; Bridge *et al*., 1997). Affected plants are prone to toppling, and consequent loss of the whole plant and bunch. Banana yield loss associated with *R. similis* and *H. multicinctus* can exceed 50% (Speijer *et al*., 1988).

Small-scale, subsistence farmers do not generally implement management practices against nematodes, apart from the occasional paring of dead rhizome tissue (Jones, 2000). The high cost of inputs remains a principal perceived reason for not using integrated pest management practices (Bagamba *et al*., 2003), although lack of knowledge on the nature and transmission of nematode pests is also a key. Alternative measures that can be easily implemented by small-scale farmers are thus required.

Endophytes are micro-organisms that spend the whole or part of their lifecycle colonizing internal tissues of their host plant, typically causing no apparent symptoms of disease (Clay and Schardl, 2002). Endophytes can be beneficial to their host in many ways, such as through stimulation of plant growth (Lu *et al*., 2000), tolerance to drought stress (Richardson *et al*., 1992), and protection against pests and diseases (Dubois *et al*., 2006; Nel *et al*., 2006; Zum Felde *et al*., 2006). Endophytes help protect their host against pests and diseases through a number of mechanisms: direct parasitism (Tjamos, 2000), competition for nutrients or colonization sites (Mandeel and Baker, 1991), induction of systemic resistance of the host plant (Vu *et al*., 2004), or production of secondary toxic metabolites (Schwarz *et al*., 2004).

Fusarium species are known to produce a wide range of toxins (e.g. T2-toxin, moniliformin, verrucarin A, cytochalasin B and enniatin B), which can cause significant mortality to juveniles of the root-knot nematode *Meloidogyne javanica in vitro* (Ciancio, 1995). Limited information is available on the nematode-inhibiting components of the fungal filtrates and on specific phytotoxins produced by endophytic *F. oxysporum*. In laboratory and screenhouse experiments, non-pathogenic endophytic *F. oxysporum* suppressed *R. similis* in banana (Niere, 2001; Dubois *et a*l., 2004; Athman *et al*., 2006), while Mennan *et al*. (2005) demonstrated the antagonistic effect of *F. oxysporum* against *Heterodera cruciferae*. Vu *et al*. (2004) and Athman *et al*. (2006) reported the *in vitro* antagonistic effect of culture filtrates of endophytic *F. oxysporum* against *R. similis*. Although Mwaura *et al*. (2009, 2010) demonstrated some *in vitro* effect of *F. oxysporum* filtrates on *P. goodeyi* and *H. multicinctus* in Kenya, the antagonistic effect of endophytic *F. oxysporum* against banana nematodes other than *R. similis* is limited. Moreover, few studies have investigated the effect of the direct environmental influences such as temperature, light, pH, etc. on the efficacy of filtrates on plant-parasitic nematodes (Burr, 1985).

In the current study, the secondary metabolites produced by endophytic *F. oxysporum* strains *Emb2.4o, Eny1.31i* and *V5w2* were assessed for activity against three key banana-parasitic nematode species, *R. similis*, *P. goodeyi* and *H. multicinctus*; and the influence of light on the antagonistic effect of strains *Emb2.4o* and *V5w2* against *R. similis* and *P. goodeyi* was determined.

Nematodes

Pure sterile cultures of *R. similis*, originating from banana in Uganda, were prepared and maintained on carrot disks in Petri dishes at the International Institute of Tropical Agriculture (IITA), Namulonge, Uganda, according to Speijer and De Waele (1997). The nematodes were harvested by rinsing the Petri dishes with sterile distilled water (SDW). *Pratylenchus goodeyi* were collected from infected banana roots in Mbarara, Uganda. After nematode extraction with tap water using a modified Baermann method (Speijer and De Waele, 1997), the nematode suspensions consisted of ~95% *P. goodeyi*. *Helicotylenchus multicinctus* were collected from infected banana roots from an experimental plot at IITA, Namulonge, and extracted as above. Nematode suspensions contained ~95% *H. multicinctus*. Nematode suspensions contained mixed stages. After collection, nematodes were concentrated and counted using triplicate 1 ml aliquots, and concentrations were adjusted to a final volume of 75 nematodes/µl.

Fungal strains and preparation of filtrates

Three *F. oxysporum* strains (*Emb2.4o, Eny1.31i* and *V5w2*), preserved in soil in 20 ml test tubes at 4°C at IITA, Namulonge, were used in the experiments. The strains have previously shown *in vitro* activity against *R. similis* (Athman *et a*l., 2006). Soil containing mycelium was transferred under sterile conditions to synthetic nutrient agar medium (SNA) (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M), 13.2 g agar/L SDW) in 55 mm diameter glass Petri dishes using a sterile needle. The fungal strains were incubated at ± 25°C with a natural photoperiod of 12 L: 12 D h for 1 week in the laboratory. Afterwards, 3 ml of autoclaved (121°C for 15 min) tap water (ATW) was added and the upper surface of the fungal culture was scraped off using a

sterile needle. Approximately 1.5 ml of ATW, containing fungal spores, was pipetted onto a haemocytometer to estimate spore density, adjusting the suspension to the required concentrations (1.8×10^6 or 5.4×10^6 conidia/ml) with SDW, before recounting to confirm concentration. In order to standardize spore production per Petri dish, 1.8×10^6 or 5.4×10^6 conidia/ml of ATW (depending on the experiment) were pipetted into 55 mm diameter Petri dishes containing 2,000 µl SNA and incubated for 1 week in the laboratory.

Liquid banana rhizome broth (BRB) was used to simulate the growth conditions under which the endophytes naturally occur. Rhizomes of 50 cm tall suckers (cv. Enyeru, genome group AAA-EA) were pared and roots removed, rinsed in tap water, cut into pieces of 1 cm^3 and 500 g of rhizomes placed in 1 L of tap water and boiled for 2 h. The mixture was passed through cheesecloth and the resultant solution adjusted to 1 L with tap water. BRB, either 50 or 100 ml depending on the experiment, was dispensed into 250 ml Erlenmeyer flasks and sterilized. After the BRB had cooled the contents of a Petri dish was transferred to the broth. For each endophyte strain, four 250 ml Erlenmeyer flasks were prepared. Inoculated flasks were shaken daily and incubated for 1 or 3 weeks (depending on experiment) in the laboratory. BRB was centrifuged at 1,613 g for 10 min. The resultant supernatant was aseptically transferred to sterile 25 ml McCartney bottles (Meyer *et al*., 2000). Flasks containing BRB alone represented control treatments. Two control treatments were used: one with the pH adjusted to equilibrate the average pH of the filtrates from each strain; one with the pH remaining unadjusted. Filtrates were stored at 4°C overnight. To avoid interference from the effect of light on the toxic metabolites and to obtain natural conditions all experiments were conducted in total darkness except for experiment 3 where the effect of light on the endophytic activities was investigated.

Experiment 1: Nematicidal effect of endophytes against R. similis, P. goodeyi *and* H. multicinctus

For each of the three strains (*Emb2.4o, Eny1.31i* and *V5w2*), the content from Petri dishes inoculated with 5.4×10^6 conidia/ml of ATW were transferred to 50 ml BRB and incubated for 3 weeks. For each strain, 1 ml filtrate was transferred to 30 mm diameter sterile glass Petri dishes. Control treatments received BRB with adjusted pH (9.2 and 9.1 in bioassays 1 and 2, respectively) or unadjusted pH (4.5 and 4.1 in bioassays 1 and 2, respectively). Petri dishes were inoculated with 75 *R. similis, P. goodeyi* or *H. multicinctus* in 100 µl tap water with three replicates per treatment. Petri dishes were organized in a completely randomized block design, and the experiment was repeated once. Petri dishes were placed in a closed metal container within an incubator at 27°C and maintained for 24 h. Afterwards, the nematodes were rinsed with 3,000 µl SDW in a 28 µm sieve and transferred to 30 mm diameter sterile glass Petri dishes, placed in a closed metal container at 27°C for an additional 24 h. Nematode mortality was assessed by recording the number of dead or live nematodes. Nematodes that were immobile after probing with a fine needle were recorded as dead (Cayrol *et al*., 1989).

Experiment 2: Nematicidal effect of endophyte concentrations against R. similis *and* P. goodeyi

The content from two Petri dishes, one inoculated with 1.8×10^6 conidia/ml of ATW of strain *V5w2* and one inoculated with 5.4×10^6 conidia/ml of ATW of strain *V5w2*, were each transferred to 100 ml BRB in separate Erlenmeyer flasks and incubated for 1 week. From each Erlenmeyer flask, 1 ml filtrate was transferred to 30 mm diameter sterile glass Petri dishes. Control treatments received BRB with adjusted pH 8.3 or unadjusted pH 6.7. Petri dishes were inoculated with 75 *R. similis* or *P. goodeyi* in 100 µl tap water, with three replicates per treatment. Petri dishes were organized in a completely randomized block design, and the experiment was repeated once. Petri dishes were maintained and nematode mortality assessed as above.

Experiment 3: Effect of two different light regimes on the nematicidal activity of endophytes

For strains *Emb2.4o* and *V5w2*, the content from Petri dishes inoculated with 5.4×10^6 conidia/ml ATW were transferred to 50 ml BRB and incubated for 3 weeks. For each strain, 1 ml filtrate was transferred to 30 mm diameter sterile glass Petri dishes. Control treatments received BRB with adjusted pH (4.5 and 4.1 in bioassays 1 and 2, respectively) or unadjusted pH (9.2 and 9.1 in bioassays 1 and 2, respectively). Petri dishes were inoculated with 75 *R. similis* or *P. goodeyi* in 100 µl tap water with three replicates per treatment. Petri dishes were organized in a completely randomized block design, and the experiment was repeated once. Petri dishes were placed in a closed metal container for 24 h for the dark treatment, and in an open metal container subjected to a photoperiod of 14 L: 10 D h for the light treatments. Nematode mortality was assessed as above.

Data analysis

In all experiments, the ratio of the number of dead nematodes to the total number of nematodes was modelled by Generalized Linear Models (McCullagh and Nelder, 1983) using the procedure GLMMIX in SAS Version 9.2 (SAS Institute, Cary, USA). Binomial distribution was assumed for the variable, logit was used as the link function, replication was defined as random effect and tests were undertaken using a significance level $\alpha = 0.05$. The analyses were conducted in three stages: (1) as a complete model, including controls and interactions, to test the hypotheses of interaction effects

Table 1. Percent mortality (mean ± standard error) of *Radopholus similis, Pratylenchus goodeyi* and *Helicotylenchus multicinctus* mixed stages (males, females and juveniles) following 24 h exposure to culture filtrates of endophytic *Fusarium oxysporum* strains *Emb2.4o, Eny1.31i* and *V5w2*.

	Treatments			
Nematode Species	*Emb2.4o*	*Eny1.31i*	*V5w2*	Nematode mean
H. multicintus	88.8 ± 4.5 a*a*	90.4 ± 3.9 a*a*	76.7 ± 7.7 a*b*	86.2 ± 4.9 c
P. goodyi	99.2 ± 0.6 b*a*	98.6 ± 1.1 b*a*	99.1 ± 0.7 b*a*	99.0 ± 0.5 a
R. similis	96.0 ± 2.3 c*a*	97.1 ± 1.7 b*a*	97.9 ± 1.4 b*a*	97.0 ± 1.3 b
Endophyte mean	96.7 ± 96.7 *a*	96.6 ± 1.6 *a*	96.2 ± 1.8 *a*	93.8 ± 2.5

In each column, means followed by the same regular letter are not significantly different (using LSD, T-test approximated distribution).
In each row, means followed by the same italic letter are not significantly different (using LSD, T-test approximated distribution).
Marginal percentages are not equal to the arithmetic cells average because the different number of observed nematodes per cell.

Table 2. Percent mortality (mean ± standard error) of *Radopholus similis* and *Pratylenchus goodeyi* mixed stages (males, females and juveniles) following 24 h exposure to culture filtrates of endophytic *Fusarium oxysporum* strain *V5w2* at two doses.

	Treatments		
Nematode Species	*V5w2* high	*V5w2* low	Nematode mean
P. goodyi	30.8 ± 13.1 a*a*	22.6 ± 9.1 a*a*	26.5 ± 8.9 a
R. similis	76.6 ± 10.2 b*a*	57.1 ± 12.6 b*a*	67.6 ± 10.5 b
Concentration mean	54.7 ± 11.7 *a*	38.4 ± 11.1 *a*	46.8 ± 7.2

In each column, means followed by the same regular letter are not significantly different (using LSD, T-test approximated distribution).
In each row, means followed by the same italic letter are not significantly different (using LSD, T-test approximated distribution).
Marginal percentages are not equal to the arithmetic cells average because the different number of observed nematodes per cell.
The high and low dose of *F. oxysporum* strain *V5w2* were made from 1.8×10^6 and 5.4×10^6 conidia/ml autoclaved tap water transferred to 100 ml banana rhizome broth in separate Erlenmeyer flasks and incubated for 1 week.

and to compare the control treatments with the endophyte treatments; (2) as a reduced model, considering only endophyte treatments and their interactions; and (3) as reduced model without considering interaction effects to estimate and compare the main effects (endophyte strain, nematode species and, the case of experiment 3, photoperiod). The tests of hypothesis under the Generalized Linear Model framework are Likelihood Ratio Tests approximated by F or T distribution; the pair wise comparisons to separate means after the rejection of the general hypotheses were done using the LSD (T test approximated distribution) because the maximum number of means in comparison was three.

Comparison of control with endophyte treatments

Dead nematodes appeared straight for *R. similis* and *P. goodeyi*, and curved for *H. multicinctus*, while living nematodes retained a sigmoid shape with some movement. Percentage mortality for the two control treatments averaged 42.6 ± 1.9%, 22.7 ± 5.5% and 40.9 ± 1.5%, for experiments 1, 2 and 3, respectively; while for the treatments they averaged 93.8 ± 2.5%, 46.8 ± 7.2% and 98.0 ± 1.5% for experiments 1, 2 and 3, respectively. In all three experiments, highly significant differences ($P < 0.001$) in nematode mortality were observed between the control and endophyte groups

Table 3. Percent mortality (mean ± standard error) of *Radopholus similis* and *Pratylenchus goodeyi* mixed stages (males, females and juveniles) following 24 h exposure to culture filtrates of endophytic *Fusarium oxysporum* strains *Emb2.4o* and *V5w2* under two light regimes.

	Treatments				
	Emb2.4o		*V5w2*		Nematode
Nematode Species	D	L	D	L	mean
P. goodyi	99.1 ± 0.5 a*a*	98.0 ± 0.7 a*a*	98.9 ± 0.6 a*a*	98.7 ± 0.6 a*a*	98.7 ± 0.3 a
R. similis	95.9 ± 1.5 b*a*	97.4 ± 1.1 a*a*	97.7 ± 1.0 a*a*	96.2 ± 1.3 a*a*	96.8 ± 0.6 b
Treatment mean	98.1 ± 0.6 *a*	97.7 ± 0.6 *a*	98.3 ± 0.5 *a*	97.4 ± 0.6 *a*	97.7 ± 1.5

In each column, means followed by the same regular letter are not significantly different (using LSD, T-test approximated distribution).
In each row, means followed by the same italic letter are not significantly different (using LSD, T-test approximated distribution).
Marginal percentages are not equal to the arithmetic cells average because the different number of observed nematodes per cell.
Light regimes were 14 L: 10 D h and 0 L: 24 D h for L and D, respectively.

of treatments. No statistical differences were observed between species within treatments on any experiment ($P > 0.05$). The difference in mortality between unadjusted pH (4.5 and 6.7) and adjusted pH (8.3 and 9.2) in BRB was not significant.

Experiment 1: Nematicidal effect of endophytes against R. similis, P. goodeyi *and* H. multicinctus

There was no significant effect between the two control treatments for each nematode species, nor between the experimental repeats (random effect) for each treatment. *H. multicinctus* was less affected by all three endophyte strains compared to *R. similis* and *P. goodeyi*, which were similarly affected by strains *Eny1.31i* and *V5w2*, while strain *Emb 2.4o* was more lethal against *P. goodeyi* (Table 1). All three strains, however, were similarly effective, except for *V5w2*, against *H. multicinctus*.

Experiment 2: Nematicidal effect of endophyte concentrations against R. similis *and* P. goodeyi

There was no significant effect between the two control treatments for each nematode species, nor between the experimental repeats for each treatment. Percentage mortality was significantly higher for *R. similis* compared to *P. goodeyi* for both treatments (Table 2). Although there was no statistical difference in nematode mortality between the two *V5w2* concentrations, the highest incubated spore concentration caused relatively higher mortality.

Experiment 3: Effect of light on the nematicidal activity of endophytic strains

There was no significant effect between the two control treatments for each nematode species, nor between the experimental repeats for each treatment. Exposure to either light or dark during incubation had no effect on percentage mortality. No significant difference was observed between the two fungal strains (*Emb2.4o* and *V5w2*), although differences were observed between *R. similis* and *P. goodeyi* with *Emb2.4o* in the dark treatment. Culture filtrates from three endophytic *F. oxysporum* strains, isolated from banana roots and rhizomes, effectively killed the three most important parasitic nematode species of East African highland bananas, *R. similis, P. goodeyi* and *H. multicinctus*, following 24 h *in vitro* exposure. Since culture filtrates were free of mycelia, the metabolites produced in the culture filtrates by the *F. oxysporum* strains were, therefore, primarily responsible for the death of the nematodes. The nematicidal effect of filtrates of *Eny1.31i, Emb2.4o* and *V5w2* on *R. similis* reflects the observations by Athman *et al*. (2006) on *R. similis.* However, our study shows that other nematode species, such as *P. goodeyi* and *H. multicinctus*, are also affected by these filtrates. The nematicidal effect was not equally effective against all three nematode species, though, with *H. multicinctus* less susceptible than *R. similis* and *P. goodeyi*. The nematicidal differences between the endophytic strains are minimal. Experiment 2 showed that the threshold levels to obtain an efficient nematicidal effect for *R. similis* and *P. goodeyi* vary for *V5w2*. In the current study, the nematicidal activity of the endophytic strains was not affected by light.

The wide range of pH among filtrates may possibly have influenced the nematicidal effect of the endophyte and on nematode behaviour. Nevertheless, Cayrol *et al.* (1989) reported that, although toxin production by fungi may be influenced by pH, the toxins act under a wide range of pH values and are independent of the culture filtrate pH. The effect of pH on nematode behaviour can be tremendous (Lee, 2002), although specific data about the effect of pH on *R. similis, P. goodeyi* and

H. multicinctus is limited. In our study, there was no significant difference in nematode mortality between the control with the adjusted pH and the control with the un-adjusted pH for the three experiments.

In the current study, the relatively high nematode mortality rates observed in the control treatments are not uncommon for such *in vitro* experiments (Niere, 2001; Athman *et al*., 2006). However, Vu (2005) obtained mortality rates below 10% in *in vitro* experiments using the *V5w2* strain, indicating that the BRB may have had an influence in the control mortality data. The BRB was prepared from banana rhizomes while nematodes occur primarily in the roots. Presumably, the physiological (e.g., osmotic) and chemical (e.g., build up of phenols) conditions of the BRB could have been traumatic to the nematodes. However, our study helps in determining the potential of endophytic *F. oxysporum* for screening purposes: differences in mortality rates were observed among strains, enabling the detection of the most effective strains. Still, the relatively high differences between the same control treatments among experiments in this study demonstrated the variation in nematode populations of the same species.

Helicotylenchus multicinctus was less sensitive to the filtrates than the other nematode species. An additional difficulty, as experienced in the current study, which often results in its omission from studies, is the difficulty in culturing and maintaining *H. multicinctus* (Gowen *et al*., 2005). Differences in mortality between *P. goodeyi* and *R. similis* were minimal except in Experiment two. No consistent differences were observed in nematicidal activity between the low and high endophytic spore concentrations. However, when mortality rates of *P. goodeyi* and *R. similis* are compared across experiments, the same high spore concentration seemed more nematicidal when incubated for a longer period and/or in more concentrated inoculation conditions. This is highlighted in Experiment two where the incubation time was 1 week in 100 ml BRB, while in Experiment one and three, with higher nematode mortalities, incubation time was 3 weeks in 50 ml BRB. Further studies are needed to establish the optimal levels for incubated spore quantities and incubation times for each endophyte. There appears to be no effect of light on the mortality rates, although this may be explained by the relatively short exposure time (14 h). Further research can focus on longer exposure times.

Although the potential of endophytic *F. oxysporum* as an alternative biological management strategy for the management of plant-parasitic nematodes was reported in this study, continued research is still needed to determine the mechanism of action of metabolites toxic to nematodes, and for the purification and identification of these toxic metabolites.

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