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Research Article

Exploration of Nematophagous Fungi from Coffee Rhizosphere Soil and their Potential as Biological Control Agents against Root-lesion and Root-knot Nematodes

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ABSTRACT

Root-lesion and root-knot nematodes are amongst the most important pathogens of coffee and can cause substantial yield losses and quality reductions. Environmental and health concerns concerning the use of chemical pesticides have increased the need for alternative management strategies against plant-parasitic nematodes. The aim of our study was to isolate and identify nematophagous fungi from nematode-infested coffee production areas and evaluate their potential as biocontrol agents. Our study was carried out in two stages: 1) fungi isolation and evaluation of their ability to affect eggs or vermiform developmental stages of root-lesion and root-knot nematodes; 2) identification of fungal isolates to species level. Eleven fungal isolates were able to affect either the nematode eggs or the vermiform developmental stages. The ability of these fungi to produce extracellular enzymes were also evaluated. This study highlights Indonesian nematode infected coffee rhizosphere soils as a rich source of nematophagous fungi, with eleven isolates showing promises to be used for integrated pest management strategies. Future work should assess field efficacy under local conditions while monitoring impacts on soil food webs and non-target organisms.

Keywords: biological control; coffee; nematode; nematophagous fungi; parasitic

INTRODUCTION

Indonesia is the fourth largest coffee producer worldwide. Plant-parasitic nematodes, including root-knot and root-lesion nematodes, are amongst the most important pathogens of coffee. They are responsible for yield losses and quality reduction in several countries, including Vietnam, India, and Indonesia. These nematodes are present in almost all coffee producing areas in Indonesia, including North Sumatra, Lampung, East and Central Java, Bali, South Sulawesi, and East Nusa Tenggara. Wiryadiputra and Tran (2008) stated that about 44.5% of coffee production area is affected by root lesion nematodes (*Pratylenchus coffeae*) and root knot nematodes (*Meloidogyne* spp.) and estimated yield losses caused by these nematodes to range from 10% to 35% depending on the nematode species, environmental conditions, and management practices (Saikai *et al.*, 2023).

Nematophagous fungi are important in the soil due to their potential benefits that can be utilized in sustainable agriculture. These fungi are members of several taxa, including Ascomycota and Basidiomycota. Different soil types can support a variety of different nematophagous fungi, particularly in soils with high nematode population densities. In a study by Renco *et al.* (2020), highest nematodes species were found in chernozem soil which were associated with forest habitat compared to stagnosol soil which were associated with agricultural soil. There is a connection between nematophagous fungi and nematode (Al-Ani *et al.*, 2022). According to Nordbring-Hertz *et al.* (2006), nematophagous fungi produce specific compounds (such as nematicidal componds or hydrolytic extracellular enzymes) based on their species and environmental circumstances, including abiotic factors and nematodes species they interact within their communities being a significant biotic factor. The effectiveness of nematophagous fungi as biological control agents against plant-parasitic nematodes is being recognized. These nematophagous fungi provide an ecofriendly alternative to the use of chemical pesticides by serving as antagonist against nematodes by parasitizing, capturing, digesting, and killing them at all developmental stages, including eggs, juveniles, and adults (Nordbring-Hertz *et al.*, 2006). The mechanism they use consists of special mycelial structures, called traps or spores, to infect nematode eggs and the vermiform developmental stages. Some nematophagous fungi also produce hydrolytic extracellular enzymes or nematicidal substrates (Chen & Dickson, 2004; Lopez-Llorca *et al.*, 2008; Nordbring-Hertz *et al.*, 2006). Extracellular enzymes, including serine proteases, chitinases, and collagenases are important to degrade the chemical structures of nematode egg shell and cuticle (Lopez-Llorca *et al.*, 2008).

The rhizosphere soil in nematode-infested area can serve as an interesting source of beneficial microorganisms such as nematophagous fungi (Indarti *et al.*, 2021; Monfort *et al.*, 2006; Xalxo *et al.*, 2013). In rhizosphere soil, nematophagous fungi can be abundant because of their interactions with plant-parasitic nematodes, especially predation. Addressing these relationships is essential to manage plant-parasitic nematode populations that pose threats to agricultural output. Therefore, the aim of our study was to find nematophagous fungi in the rhizosphere soil from infested coffee plantations and assess their suitability as biocontrol agents of root-lesion and root-knot nematodes that are important coffee pathogens.

MATERIALS AND METHODS

Rhizosphere Soil Collection and Isolation of Fungi

Rhizosphere soil samples were collected from depth of 5−30 cm around the roots of coffee plants from nematode-infested plantation located in Afdeling Malangsari, Afdeling Blawan, and Afdeling Kalisat-Jampit, Bondowoso Regency, East Java Province. A total of 10−15 samples were taken at each location. Fungi were isolated from the rhizosphere soil samples using the dilution plate method; i.e. each rhizosphere soil sample was diluted in sterile water to about 100 times, and 1 mL of suspension was placed on potato-dextrose agar (PDA) in Petri dishes and incubated at 25 °C in an incubation chamber for 7−10 days. Single colonies formed on PDA were transferred to fresh PDA to obtain pure isolates (Indarti *et al.*, 2010).

Nematode Populations

Meloidogyne incognita population was maintained on susceptible tomato plants, cv Kaliurang, grown in sterile soil substrate in pots and a glasshouse while the *Pratylenchus coffeae* population was maintained on carrot discs in the laboratory (Boisseau & Sarah, 2008). The *M. incognita* population was obtained from the collection of the Nematology Sub-Laboratory, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, while *P. coffeae* population isolated from infected coffee plants. Eggs masses of *M. incognita* obtained from galled tomato roots were shaken in a 0.05% NaOCl solution to remove the gelatinous matrix surrounding the eggs (Hussey & Barker, 1973), gently washed with distilled water and maintained at 25 °C before tests. *P. coffeae* vermiforms, a mixture of second-stage (J2), third-stage (J3), fourth-stage (J4) and adults, were obtained from sliced carrot cultures and maintained at 25 °C before tests.

Harvesting *P. coffeae* was done by choping discs into small pieces and nematodes were extracted by using the Whitehead tray technique with modification (Southey, 1986).

Effect of Fungal Filtrates on Eggs of *M. incognita* and Developmental Stages of *P. coffeae*

Two hundred microliter of fungal spore suspension containing 10^6 spores/mL were transferred to 24 multiwell plates and incubated at 30 °C. Each well plate contained 50 *M. incognita* eggs, and 50 *P. coffeae* vermiform individuals. Each treatment was plotted randomly to each well plate. The ability of the fungal isolates to infect either *M. incognita* eggs or *P. coffeae* vermiforms was observed each 24 hours during 7 days. Each test consisted of three replicates.

Morphological and Molecular Identification of Fungal Isolates

Fungal isolates were identified to genus level based on their morphological characters using the descriptions by Barnett & Hunter (1998) and Domsch *et al.* (1993). Ribosomal gene sequences in the ITS region of 10 fungal isolates with parasitizing ability on either root-knot nematodes eggs and root-lesion nematodes vermiform stage were analysed to identify isolates to species level. Genomic DNA of the fungal isolates was extracted using modified cetyltrimethylammonium bromide (CTAB) techniques (Zhou *et al.*, 2007). The extracted DNA was then amplified using a BioRad T-100 thermal cycler with universal primer pair of ITS 1 (5'-TCCGTAGGTGAACCTGCGG3') and ITS 4 (5'-TCCTCCGCTTAT TGATATGC-3'). Amplified DNA was visualized on a 1% agarose gel. Bands indicating varying concentrations of the PCR products were then cut, removed, and the sent for sequencing using an Applied Biosystems DNA Sequencer (model ABI 3700). NCBI BLAST analysis was used to compare the resultant sequences with sequences available in the GenBank database.

Ability of the Fungal Isolates to Produce Extracellular Enzymes

Fungal isolates ability to produce protease and chitinase was determined using an agar medium containing 2% skimmed milk (for the protease test) and 2% colloidal chitin (for the chitinase test). The solid media were inoculated using fungal isolates and incubated at room temperature for 4−7 days. After this incubation period, clear zones formed around the fungal colonies were measured. The hydrolysis ability of each fungal isolate was calculated as the ratio of the size of the clear zone (halo) to the diameter pf the fungal colony. The extracellular enzymatic activity was expressed as (1-C/H) with C = the diameter of the fungal colony and H = the diameter of the halo caused by substrate degradation.

RESULTS AND DISCUSSION

Eleven isolates of nematophagous fungi found from rhizosphere soil of surveyed coffee plantations (Figure 1) were able to affect *M. incognita* eggs or *P. coffeae* vermiform (Table 1). Seven isolates had >50% parasitism ability where four isolates could affect *P. coffeae* vermiforms and five isolates could affect *M. incognita* eggs. Isolate 5b and 3 had ability to affect both nematode eggs and vermiforms (Figure 1). Infected nematode eggs or vermiform showed fungal hyphae growth (Figure 2 and 3). Nematophagous fungi with ≥50% parasitism ability are often tolerant against many environmental condition making them potential agents for managing plant parasitic nematodes (Indarti *et al.*, 2010).

Extracellular enzymes produced by tested fungal isolates are shown in Table 2. Ten of eleven fungal isolates had abilities to penetrate and infect tested nematode development stages by using proteases. Protease is necessary for breaking down structural proteins that shield nematodes from exogenous hazards (Zhang *et al.*, 2024). Nematophagous fungi pathogenicity depend on their produced hydrolytic enzymes. By dissolving important structural elements like chitin and the proteins in eggshells vermiform nematodes cuticles. In our study, ten of the nematophagous fungal isolates produced hydrolytic extracellular proteases (Table 2). The highest production of proteases was from isolate HSA1. Degradation from both vermiform nematodes or eggshells were observed after treatment with crude proteases (Figure 4 and 5).

Fungal crude enzymes contain substances that are toxic and destructive against nematodes. These substances can be extracellular enzymes or secondary

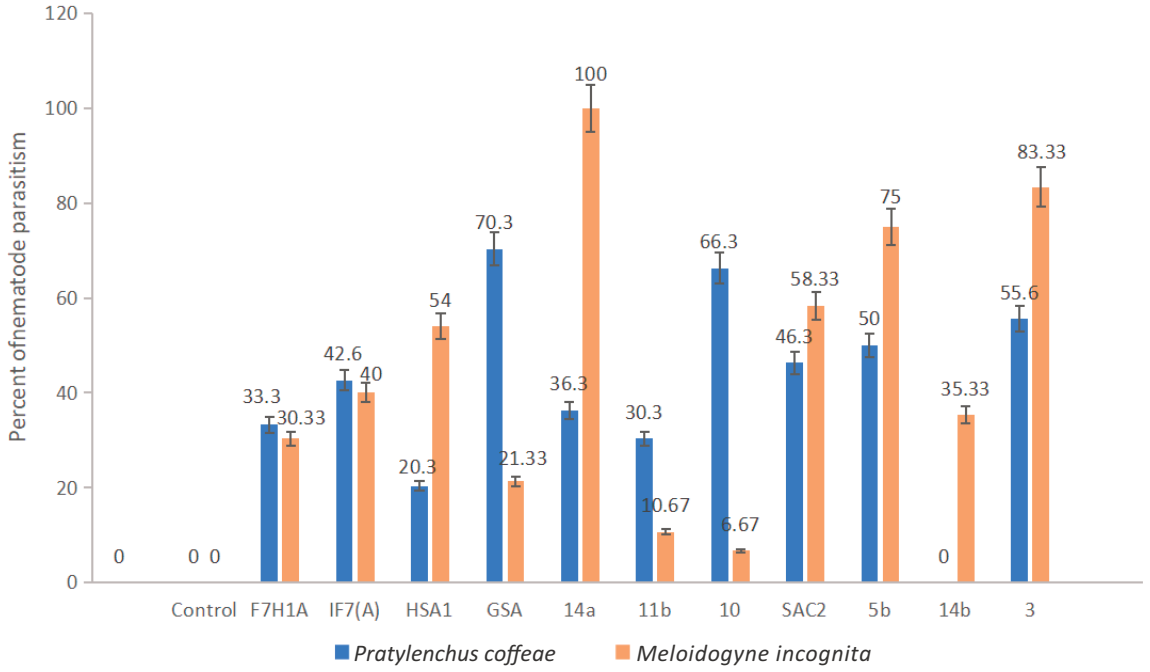

Figure 1. Effect of fungal isolates on *Meloidogyne incognita* egg hatching and the *Pratylenchus coffeae* vermiform viability 7 days after incubation.

Table 1. Isolate code, species name and isolate, locality and isolation source of the fungi examined on their ability to infect *Meloidogyne incognita* and *Pratylenchus coffeae*

Isolate Code	Species	Isolate	Locality	Isolation Source
14a	*Aspergillus flavus*	AF-25	Malangsari	Soil
IF7(A)	*Aspergillus flavus*	AF-25	Plalagan, Blawan	Soil
F7H1A	*Aspergillus* sp.	CBPFEAs-4	Plalagan, Blawan	Soil
10	*Beauveria brongniartii*	EPF3	Malangsari	Soil
HSA1	*Clonostachys* sp.	CWG1(1)	Plalagan, Blawan	Soil
11b	*Fusarium oxysporum*	KD3	Malangsari	Soil
SAC2	*Penicillum janthinellum*	CCG1(1)	Plalagan, Blawan	Soil
GSA	*Penicillium* sp.	BRO-2013	Plalagan, Blawan	Soil
3	*Trichoderma asperellum*	unilaGT	Malangsari	Soil
5b	*Trichoderma hamatum*	bai3	Malangsari	Soil

Table 2. Qualitative test of fungal extracellular enzyme on skim milk agar chitine colloidal

Isolate code	Ratio of halo zone to colony diameter	
	Protein	Chitin
14a	1.42	-
IF7(A)	1.16	-
F7H1A	1.14	-
10	1.78	-
HSA1	4.78	-
11b	-	-
SAC2	1.42	-
GSA	1.52	-
3	1.75	-
5b	1.40	-
14b	1.23	-

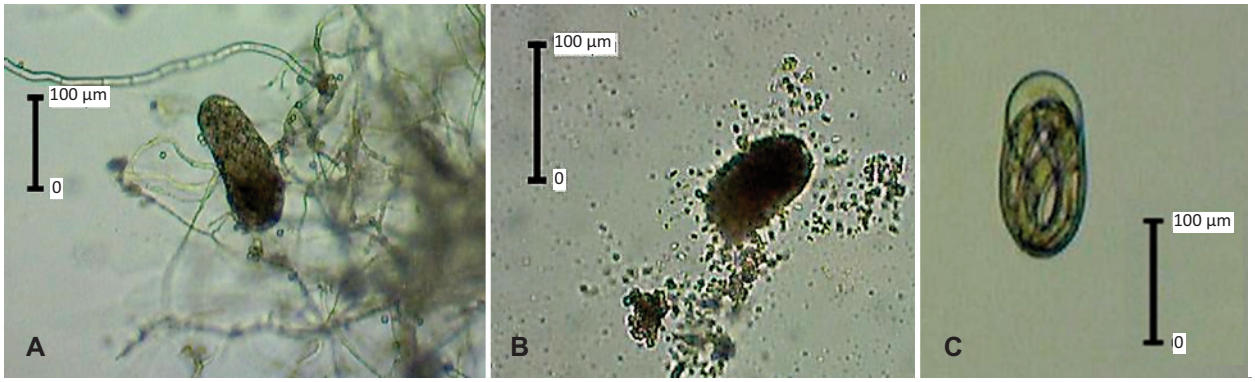

Figure 2. Effect of fungal spore on eggs of *Meloidogyne incognita* (A) isolate 14a; (B) isolate SAC2; (C) control.

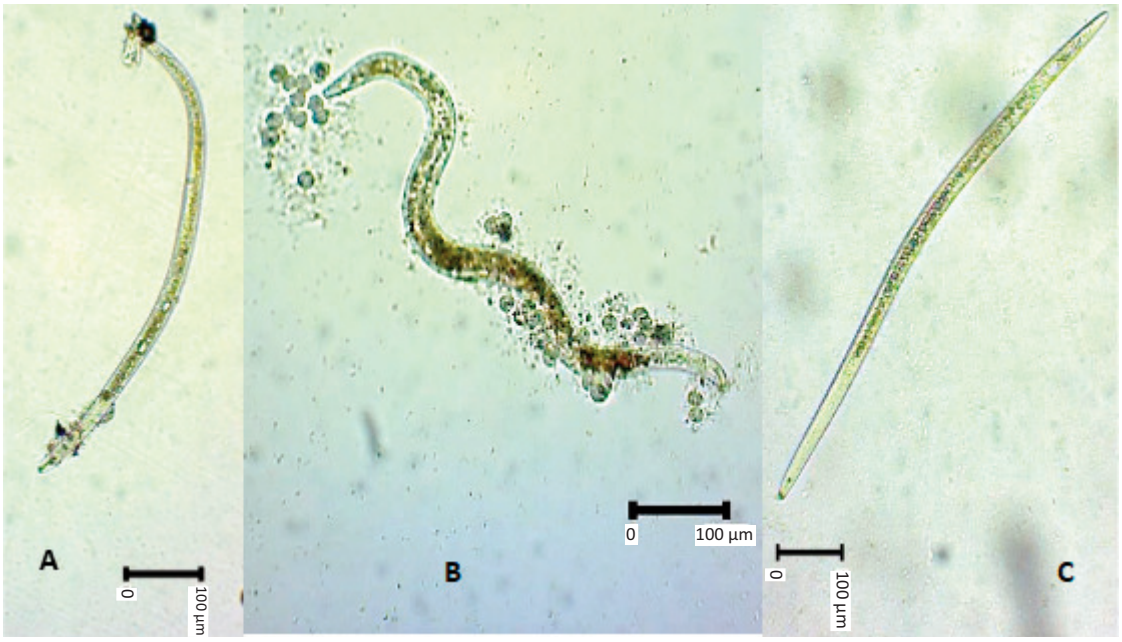

Figure 3. Effect of fungal spore on vermiforms of *Pratylenchus coffeae* (A) isolate 5b; (B) isolate 14a; (C) control.

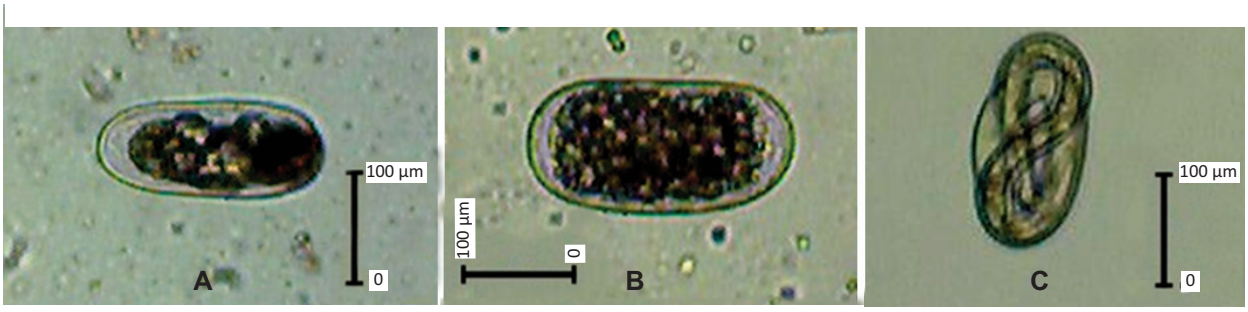

Figure 4. Effect of fungal spore suspensions on *Meloidogyne incognita* eggs, (A) isolate F7H1A; (B) isolate HSA; (C) control.

metabolites (Qureshi *et al.*, 2012). Hydrolytic extracellular enzymes also determine the level of virulence of nematophagous fungi (Zhang *et al.*, 2024). The activity of these enzymes produced by nematophagous fungi correlates with the main components of the nematode cuticle (Huang *et al.*, 2004).

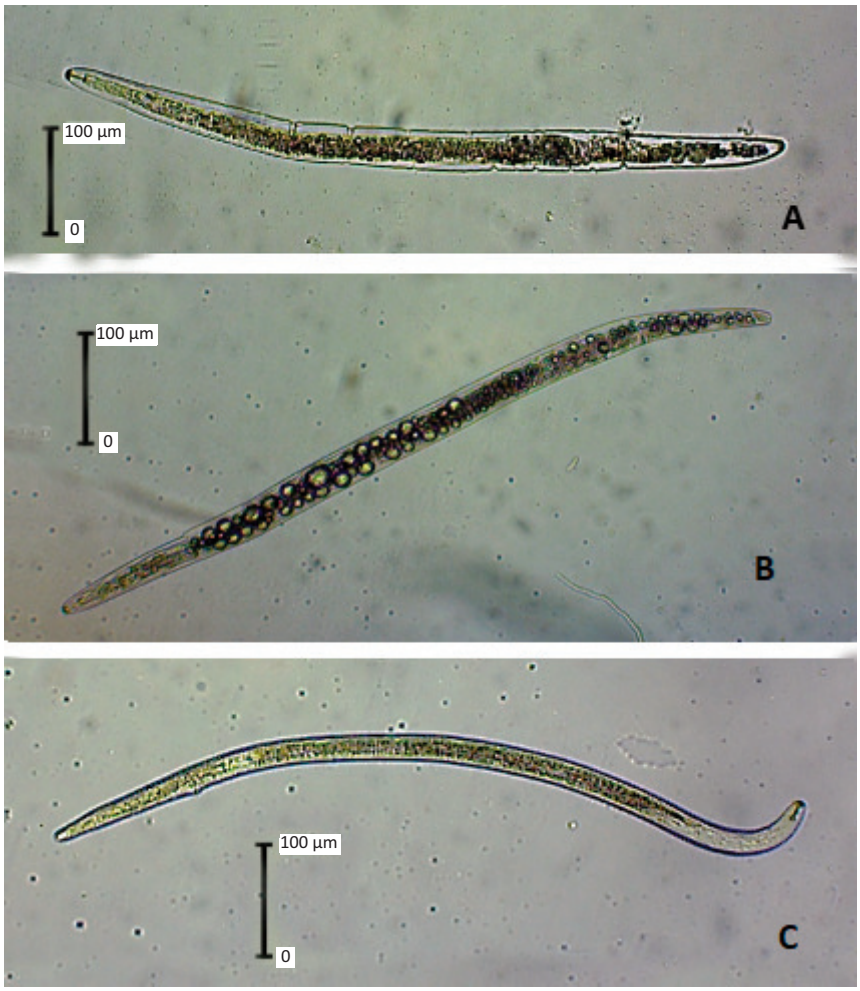

Figure 5. Effect of the fungal spore suspensions *Pratylenchus coffeae* vermiforms (A) isolate HSA; (B) isolate SAC2; (C) control.

Hydrolytic extracellular enzymes are a key component of the mechanical and biological approaches used by nematophagous fungi to infect and kill plant-parasitic nematodes. Proteases and chitinases are two of the most significant enzymes. In this study, eleven nematophagous fungal isolates only produce proteases. Proteases are essential for breaking down nematodes' protein-rich structural barriers, such as their cuticle and eggshell, which are primarily made of proteins, glycoproteins, and vitellin. Proteases help fungal structures (like hyphae or spores) enter the nematode body resulting in successful infection and host's death. Thus, these enzymes are regarded as essential factors that influence fungal virulence and the effectiveness of biocontrol.

Eleven fungal isolates from our study were able to affect *M. incognita* vermiform while ten fungal isolates affected *P. coffeae* vermiform. Sequence results of these isolates were blasted against data in GenBank (Table 3). Isolate effects that were higher than 50% may indicate that they (isolates 5b and 3) are good candidates for nematode biocontrol agents.

CONCLUSION

Our study successfully isolated 11 fungal isolates and identified 10 nematophagous fungi from the rhizosphere soil of nematode-infested coffee plantations with the ability to affect *M. incognita* eggs and *P. coffeae* vermiforms. The nematophagous fungal isolates infection rates and extracellular enzyme

Table 3. Isolate code of the fungi examined on their ability to infect *Meloidogyne incognita* and *Pratylenchus coffeae* with top blast percentage identity, sequence length, and accession number in Genbank.

Isolate Code	Species	Top blast percentage identity (%)	Sequence length (bp)	Genbank accession number
14a	*Aspergillus flavus* AF-25	99.83	619	PP922364.1
IF7(A)	*Aspergillus flavus* AF-25	99.50	619	PP922364.1
F7 H1A	*Aspergillus* sp. BPFEAs4	99.67	648	MW882244.1
10	*Beauveria brongniartii* EPF3	99.08	542	OP856951.1
HSA1	*Clonostachys* sp. CWG1(1)	99.13	594	KM268679.1
11b	*Fusarium oxysporum* KD3	100.00	547	PQ285819.1
SAC2	*Penicillum janthinellum* CCG1(1)	99.66	615	KM268697.1
GSA	*Penicillium* sp. BRO-2013	99.83	791	KF367521.1
3	*Trichoderma asperellum* unilaGT	99.01	609	LC650154.1
5b	*Trichoderma hamatum* bai3	99.82	575	MK377311.1

productions were indication of their potential as effective biological control agents against plant-parasitic nematodes. Isolate 5b (*Trichoderma hamatum*) and 3 (*Trichoderma asperellum*) showed promising results as nematodes biological control agents.

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