Official publication of Pakistan Phytopathological Society
Pakistan Journal of Phytopathology
ISSN: 1019-763X (Print), 2305-0284 (Online)
http://www.pakps.com

BIOCONTROL POTENTIAL OF NEMATODE-TARGETING FUNGI FROM COFFEE PLANT RHIZOSPHERE AGAINST *PRATYLENCHUS COFFEAE* ROOT LESION NEMATODE

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A B S T R A C T

Environmentally friendly control techniques need to be developed to overcome the negative impact of nematicides in controlling the root-lesion nematode *Pratylenchus coffeae*. This study aimed to isolate nematode-trageting fungi and evaluating them by performing pathogenicity tests on *P. coffeae*. The results revealed five selected isolates with > 50% mortality out of the 58 tested isolates: PIIIB0402b, PIVB0402a, PIIB0402b, PIVA0302b, and PIVA0402b. Phenotypic character identification and analysis using ITS rDNA regional molecular markers confirmed that four isolates belonged to *Penicillium*, while one isolate remained unidentified at the genus level but was considered as *Ascomycota*. Among the five selected isolates, PIIIB0402b and PIVB0402a showed the most potential to be developed as biocontrol agents, with mortality rates of 77.5% and 67.0%, respectively. However, further research is required to assess the efficacy and feasibility of these antagonistic fungus isolates as a biocontrol method for *Pratylenchus* nematodes on coffee plants.

Keywords: coffee plant, root-lesion nematode, *Pratylenchus coffeae*, nematode-targeting fungi, biocontrol agents.

INTRODUCTION

Plant-parasitic nematodes pose a significant threat to the production of various agricultural commodities worldwide, including plantation crops like coffee. Some key nematodes affecting coffee plants are the root-lesion nematode (*Pratylenchus coffeae*), burrowing nematode (*Radopholus similis*), root-knot nematode (*Meloidogyne sp.*), and reniform nematode (*Rotylenchulus reniformis*) (Wiryadiputra and Tran, 2008; Orisajo and Fademi, 2012). *Pratylenchus coffeae* is found in coffee plantations across several regions worldwide, including Indonesia, where it has been responsible for yield losses of up to 78% (Wiryadiputra and Tran, 2008). *Pratylenchus coffeae* infects coffee plants through the roots, infiltrating the epidermal tissue and root cortex, leading to the formation of longitudinal lesions that disrupt nutrient transport (Inomoto and Oliveira, 2008). Consequently, infected plants show symptoms such as yellowing leaves, wilting, and ultimately, plant death (Nelson et al., 2002).

Biological control (biocontrol) using nematode antagonistic fungi is an essential approach to reduce synthetic chemical pollution while effectively managing plant-parasitic nematodes. Implementing biocontrol in coffee plants involves harnessing indigenous microbes, particularly antagonistic fungi, in the plant root area known as the rhizosphere. The antagonistic microbes found in the rhizosphere of infected plants exhibit tremendous potential as biocontrol agents. These microorganisms have adapted to survive in the suboptimal environments that serve as habitats for parasitic nematodes (Inomoto and Oliveira, 2008; Indarti et al., 2021; Widianto et al., 2021).

Research on *in situ* exploration of fungi as a biocontrol for *P. coffeae* on coffee plants in Indonesia has not been reported yet. Previous studies focused on utilizing the fungus *Paecilomyces lilacinus* strain 251 (PL 251), which showed the ability to suppress *P. coffeae* populations on coffee plants (Wiryadiputra, 2002). *P. lilacinus* has been

Submitted: June 28, 2023
Revised: August 17, 2023
Accepted for Publication: December 08, 2023
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reported can significantly decrease the root gall and eggmass of *Meloidogyne incognita* (Khalil et al., 2012). Building on these findings, it is essential to conduct a comprehensive study concerning fungal exploration in the rhizosphere soil of coffee plants to identify new, effective biocontrol agents for *P. coffeae*. Therefore, this research aims to obtain and identify potential isolates as biocontrol fungi against *P. coffeae* in the rhizosphere soil of coffee plants and to determine the antagonistic mechanisms of these fungi against *P. coffeae*.

MATERIALS AND METHODS

Fungal culture: The fungal culture was isolated from the rhizosphere of both healthy and nematode-infected plants in coffee plantations located in Plalangan, Malangsari, Kalisat, Bondowoso Regency, East Java. Isolation was conducted using the pour plate method (Frackland et al., 1995) with dilutions of 10^{-3} and 10^{-4} on Potato Dextrose Agar (PDA) media supplemented with chloramphenicol to inhibit bacterial growth (Dhingra and Sinclair, 1985).

Nematode test: *Pratylenchus coffeae* was isolated and extracted from infected coffee roots using the whitehead-tray method (Whitehead and Hemming, 1965) at the same location as the source of the fungal culture. Nematodes were propagated using the carrot disc culture method (Boisseau and Sarah, 2008). For each treatment, the 30 tested nematodes consisted of a mixture of Larvae 2 (L2), Larvae 3 (L3), and Larvae 4 (L4) that have been identify by their morphological characters especially their reproductive organs development

Selection and confirmation of biocontrol fungal isolates: The fungal isolates were selected using an *in vitro* pathogenicity test (Qureshi et al., 2012; Sun et al., 2021). Two hundred microlitre fungal spore suspension with a density of 10^6 spores/ml was exposed to 30 tested nematodes in one treatment unit in a multiwell plate with three replications. Incubation was carried out for 7 days at 27°C, and the percentage of mortality was calculated. The confirmation test was carried out using the same method with four replications. The results of this confirmation test were used to obtain 5 selected isolates with the highest mortality percentage above 50% (Indarti et al., 2010).

The liquid culture filtrate test of selected fungal isolates: Selected fungal isolates from the confirmation test were cultivated in 100 ml of Potato Dextrose Broth (PDB) media in 200 ml Erlenmeyer flasks for 7 days. After centrifugation at 3000 rpm for 25 minutes, the supernatant containing the fungal culture filtrate was separated from the fungal cells. The filtrate was then clarified by passing it through filter paper and Whatman paper to obtain a spore-free filtrate. For the test, 200 µL of the filtrate was added to 30 *P. coffeae* nematodes in a multiwell plate, with four replications. As controls, both aquadest and PDB were used. The plates were incubated at 27°C for 7 days, and the percentage of dead nematodes was recorded as mortality (Qureshi et al., 2012; Bhat and Wani, 2012; Indarti et al., 2010).

The ability of selected isolates to produce protease and chitinase extracellular enzymes: Tests were conducted using solid media containing skimmed milk and colloidal chitin. A solid medium containing 2% skimmed milk (for the protease test) and 2% colloidal chitin (for the chitinase test) was inoculated by puncturing with selected fungal isolates. The plates were then incubated at room temperature for 3-5 days. The ability of the fungal isolates to produce protease and chitinase enzymes was observed by the formation of a clear zone around the colonies growing on the respective medium (Warcup, 1950; Abe et al., 2015). The hydrolysis capacity of each isolate on the skimmed milk and chitin medium, used as the sole carbon source, was calculated based on the ratio between the size of the diameter of the hydrolysis zone around the colony and the diameter of the isolate growing on the medium.

The crude ethyl acetate extract test of selected fungal isolates against *Pratylenchus coffeae*: The test was conducted using a modified method based on Akshaya et al. (2021), where the crude extract obtained from 200 µL of ethyl acetate was added to 30 *P. coffeae* nematodes. Observations were carried out for 7 days, and the percentage of nematode mortality was calculated on the 7th day.

Selected isolate identification: Fungi were identified using phenotypic characteristics, including the morphological traits of colonies, hyphae, and conidia, referring to the Pictorial Atlas of Soil and Seed Fungi (Watanabe, 2010). For molecular analysis, the ITS1F and ITS4R primers were used to amplify the ITS rDNA region. Sequence similarity was analyzed using the BLAST program, and the phylogenetic tree was reconstructed using the MEGA 5.2 program with the Neighbor-Joining Tree algorithm (Saitou and Nei, 1987).

RESULTS AND DISCUSSION

Fungal isolation in the rhizosphere soil of the coffee plant: The isolated fungi from the rhizosphere soil of coffee

plants (Table 1) showed no significant difference in the number of fungal isolates obtained from the rhizosphere soil of healthy and infected coffee plants. However, the number of isolates obtained from the Plalangan location was higher compared to those from Malangsari and Kalisat. The Malangsari location is situated at an altitude of approximately 595 m above sea level (asl) with andosol and latosol soil types. The Plalangan afdeling coffee plantation is located not far from the Kalisat afdeling. Both plantations have similar conditions in terms of soil type and altitude, ranging from 1100 to 1534 m asl with andosol, regosol, and alluvial soil types.

Table 1. Number of fungal isolates at various coffee planting locations.

Location	Number of fungal isolates		Amount
	Healthy plant rhizosphere	Infected plant rhizosphere	
Malangsari	8	8	16
Plalangan	13	19	32
Kalisat	4	5	9
Total	25	32	58

There were differences in the number of sample points from each location based on the number of sample points infected by nematodes. The type of soil and the number of sample points carried out were considered to be the causes of the difference in the number of fungal isolates obtained. Hence, the number of fungal isolates obtained at the Plalangan Afdeling location is the highest compared to other locations. This finding is in line with the research of Indarti et al. (2021), who found that soils infected with nematodes are a source of antagonistic fungi against nematodes that have the potential to be developed as biocontrol agents.

Pathogenicity test of fungal isolates against *Pratylenchus coffeae:* Based on the selection of fungal isolates (Figure 1), 15 isolates (PIIIB0402b, PIIA0302, PIVA0403c, PIVA0302b, KIB0301a, PIVA0403a, H10202b, KIA0303a, PIVA0402b, KIB0303b, PIIB0402b, PIVB0402a, PIA0302a, PIVB0402c, and PIIIB0403b) were obtained, showing varying percentages of nematode mortality. According to the criteria for effective nematode biocontrol (El-Ghany et al., 2012), fungal isolates causing nematode mortality greater than 50% were selected for further testing. Therefore, isolates PIVA0402b, PIVA0302b, PIIB0402b, PIVB0402a, and PIIIB0402b were chosen for further investigation.

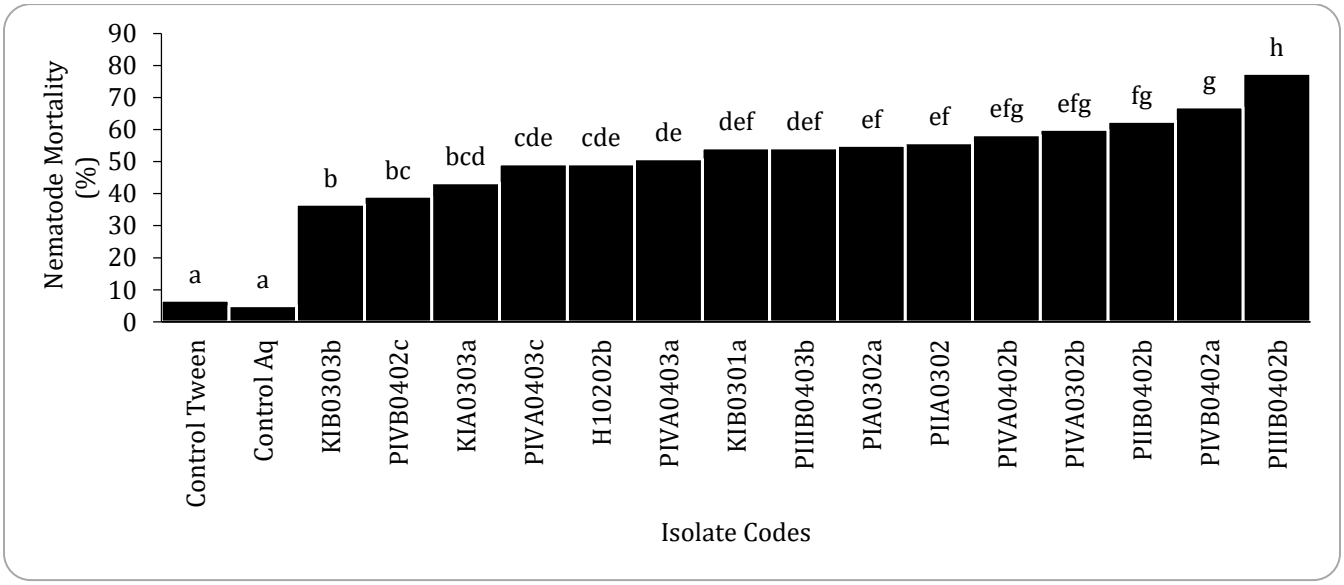

Figure 1. Confirmation test of fungal isolates on nematode mortality. Different letter notation indicates significantly different treatments according to ANOVA (α = 0.05%).

Toxicity test of liquid culture filtrate of the selected fungal isolates: The toxicity test of the liquid culture filtrate of selected fungal isolates (Figure 2) demonstrated a significant effect on the mortality rate of *P. coffeae*. The control treatment, using PDB media, did not show a significant difference, indicating that the media itself was not toxic to nematodes. Among the tested fungal isolates, PIIIB0402b exhibited the highest percentage of nematode

mortality (100%), while PIVA0402b showed the lowest (71.67%). Additionally, the treatment using the liquid culture filtrate of isolate PIVA0402b did not show a significant difference from that of PIVA0302b. Previous studies have reported that the liquid culture filtrate or supernatant of nematode biocontrol fungi can cause nematode death in in vitro tests (El-Ghany et al., 2012; Regaieg et al., 2010; Qureshi et al., 2012).

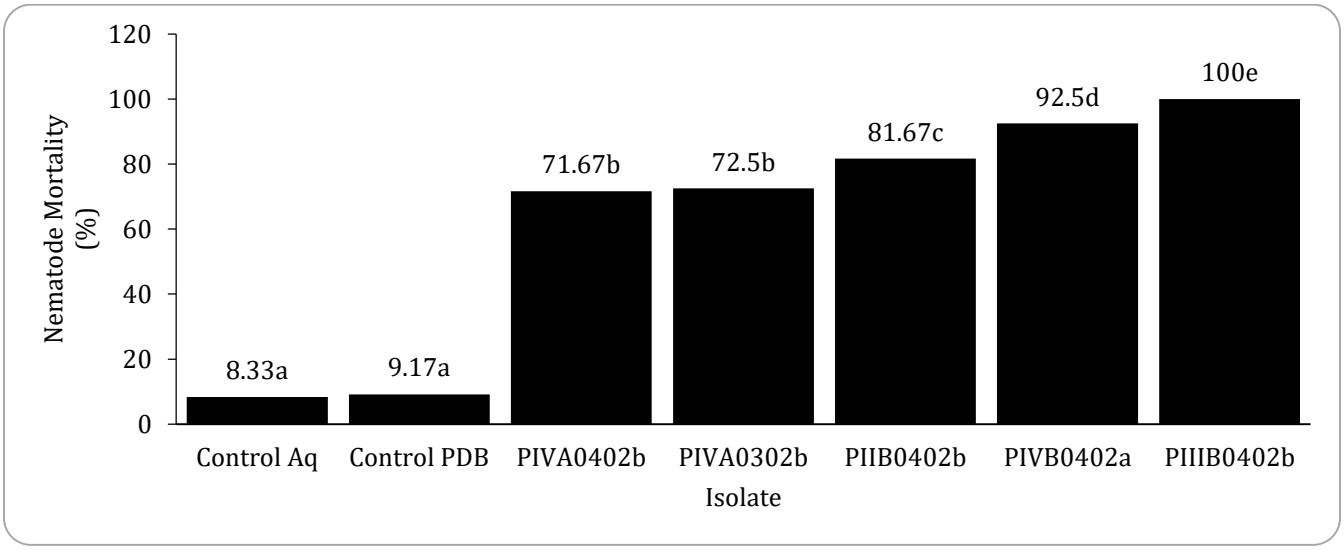

Figure 2. Toxicity test of selected fungal isolates liquid culture filtrate on nematode mortality. Values followed by the same letter were not significantly different according to ANOVA (α = 0.05%).

Qualitative tests of selected fungal isolates produced protease and chitinase extracellular enzymes: The five selected isolates were found to form clear zones on skimmed milk agar media, with the largest clear zone ratio observed for isolate PIIIB0402b. However, no clear zones were formed on chitin colloidal media (Figure 3). These results indicate that the five selected isolates can produce protease enzymes but do not produce chitinase enzymes. Protease enzymes are known as extracellular hydrolytic enzymes involved in the degradation of the nematode cuticle layer (Pandit et al., 2014; Hu et al., 2020).

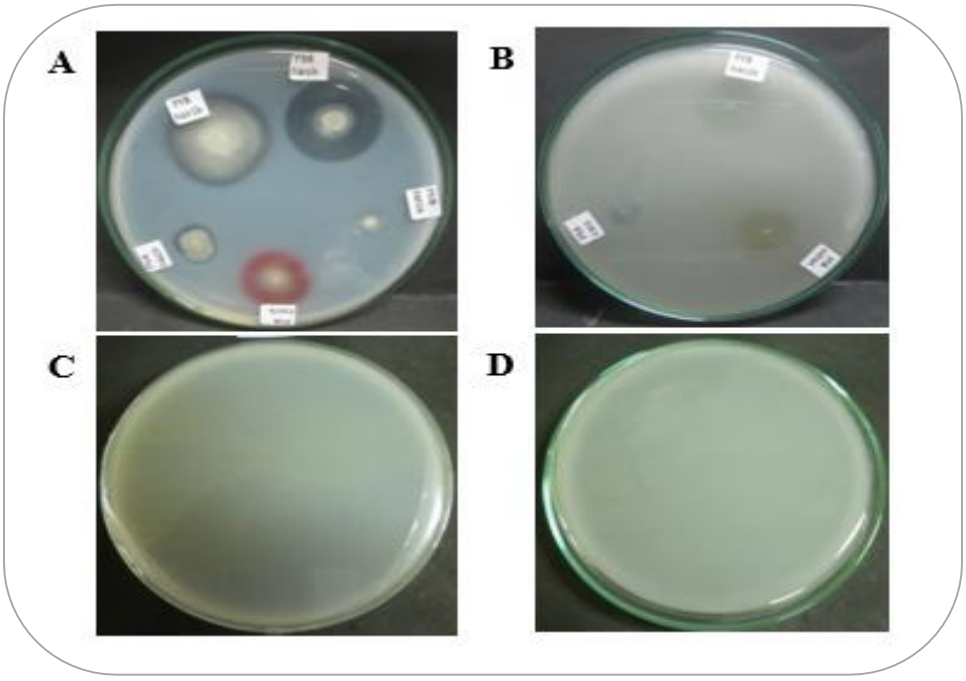

Figure 3. Protease (A) and chitinase (B) extracellular enzyme potency test, skimmed milk agar control (C), and media control with colloidal chitin (D).

Toxicity test of crude ethyl acetate extracts of selected fungal isolates: The crude extract of secondary metabolites from the selected isolates demonstrated a significant effect on nematode mortality (Figure 4). The percentage of nematode mortality caused by secondary metabolite extracts exceeded 50%. However, the results showed no significant difference among the five secondary metabolite extract treatments, but there was a significant difference between the control treatments with distilled water and PDB media. Based on these findings, the toxic mechanism of secondary metabolites is considered one of the antagonistic mechanisms employed by the selected fungal isolates against parasitic nematodes.

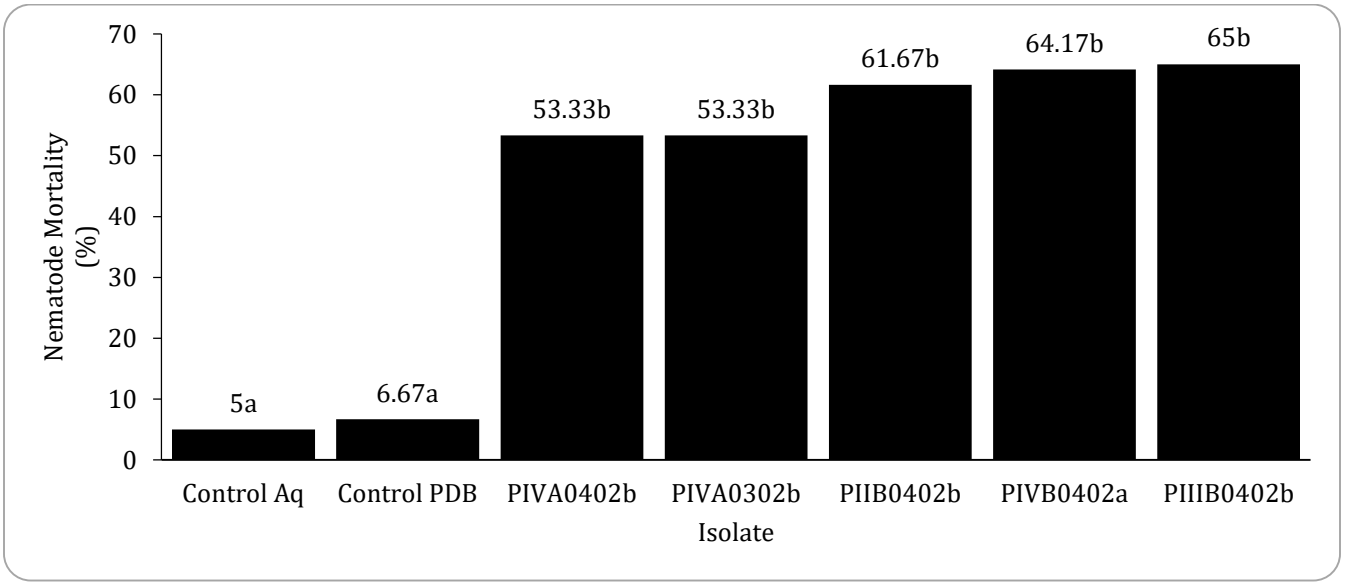

Figure 4. Toxicity test of crude ethyl acetate extract on nematode mortality. Values followed by the same letter were not significantly different according to ANOVA (α = 0.05%).

Selected fungal isolate identification: Based on the phenotypic characters, the four selected isolates (PIIIB0402b, PIVB0402a, PIIB0402b, and PIVA0302b) were identified as *Penicillium*, while PIVA0402b had not been identified but was considered Ascomycota (Figure 5). Additionally, molecular analysis using nitrogenous base sequences in the ITS rDNA region was performed using BLAST similarity analysis (Table 2) and phylogenetic analysis using phylogenetic tree reconstruction (Figure 6).

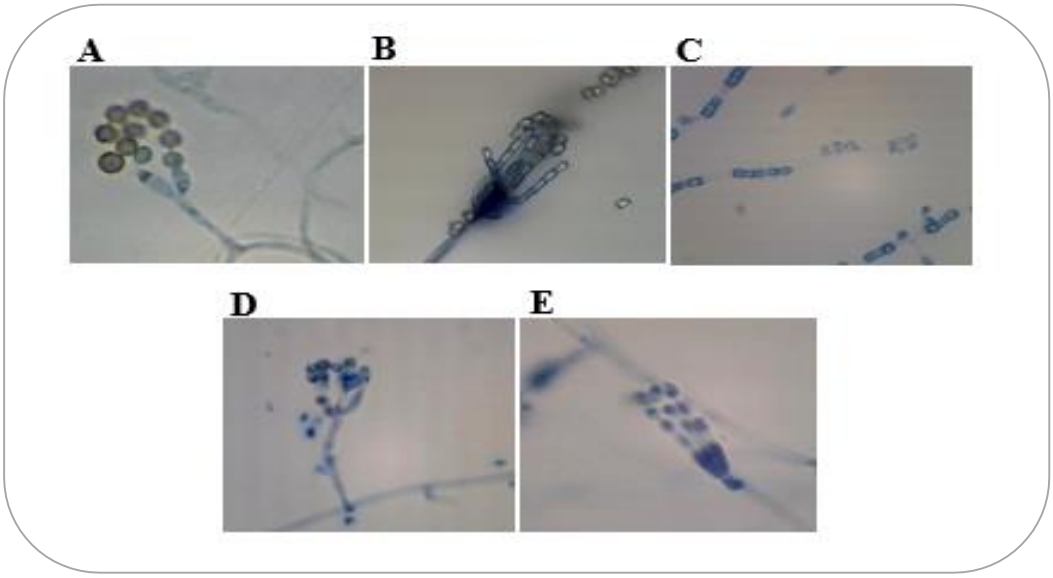

Figure 5. The hyphae and conidia of the five selected isolates (A.PIIIB0402b; B. PIVB0402a; C. PIVA0402b; D. PIIB0402b; E. PIVA0302b).

Table 2. The similarity of the selected isolates.

Isolate code	Similar Strains		
	Accession Number	Species Name	% Similarities
PIIIB0402b	JN246047.1	*Penicillium rubidurum*	97 %
PIVB0402a	HM469415.1	*Penicillium steckii*	98 %
PIIB0402b	KM268714.1	*Penicillium janthinellum*	99 %
PIVA0302b	JN246047.1	*Penicillium janthinellum*	99 %
PIVA0402b	HM037655.1	Uncultured soil fungus clone A23	97%

Out of the five selected isolates, four belonged to the *Penicillium* genus, including *P. rubidurum*, *P. steckii*, and *P. janthinellum*, while one isolate was classified as Ascomycota. *Penicillium* has previously been reported to be isolated and identified in rhizosphere soil samples from Arabica and Robusta coffee plantations. Other types of fungi that have also been reported in these soil samples include *Aspergillus*, *Fusarium*, *Cladosporium*, *Mucor*, *Trichoderma*, *Eurotium*, *Wallenia*, and *Rhizopus* (Velmourougane, 2006).

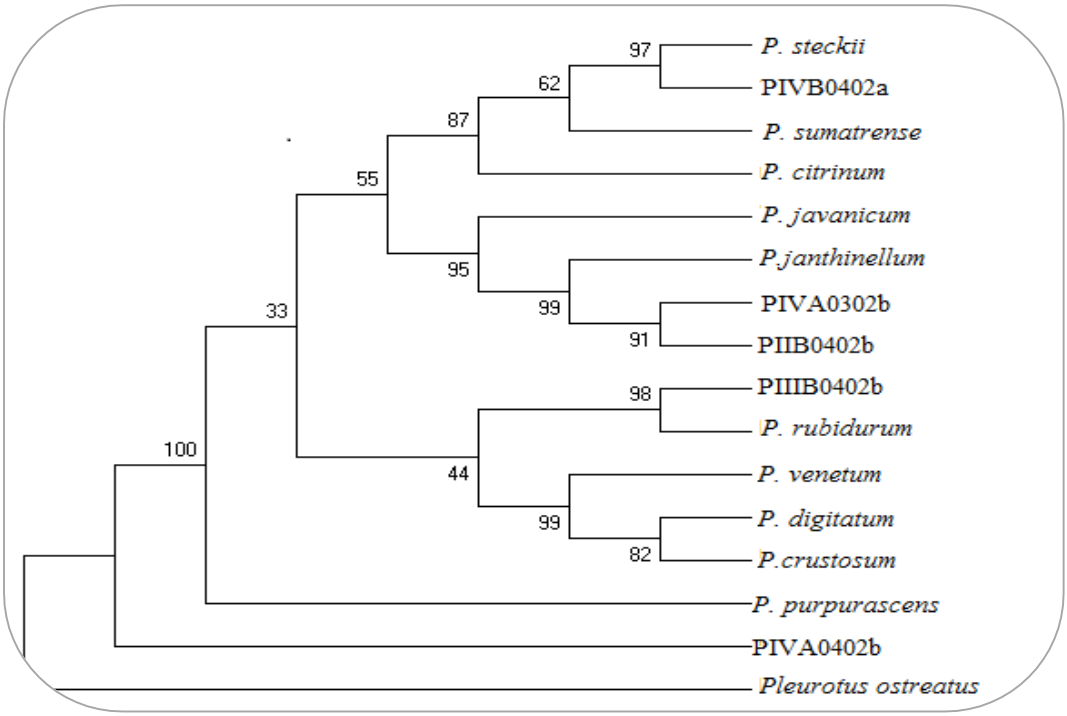

Figure 6. The phylogenetic tree of the five isolated ITS areas was selected using the MEGA 5.2 program and the Neighbor-Joining algorithm.

The phylogenetic tree (Figure 6) showed the suitability of the results with sequence similarity analysis at Genbank using the BLAST program. Isolates PIVB0402a, PIIB0402b, PIVA0302b, and PIIIB0402b were identified as *P. steckii, P. janthinellum*, *P. janthinellum, and P. rubidurum*, respectively. Isolate PIVA0402b cannot be identified yet but is suspected to be an Ascomycota because it shows a closer kinship with *Penicillium* (Ascomycota) compared to *Pleurotus ostreatus* (Basidiomycota) as an outgroup. Species from Basidiomycota are used as an outgroup because they are the phylum closest to Ascomycota. Saprotrophic fungi from Ascomycota and Zygomycota have possibility control of insects and nematodes by increasing the abundance of entomopathogenic and nematophagous species in longterm control (Matecka et al., 2015).

Several studies have reported that *Penicillium* can act as a biocontrol against *Meloidogyne* spp. (Quereshi et al., 2012; John and Wright, 2011) and the golden cyst nematode *Globodera rostochiensis* (Indarti et al., 2010). *Penicillium* is also reported to be able to produce nematicidal compounds for nematodes (Li and Zhang, 2013; Li et al., 2007). These compounds are prenipratynolene, brefeldin A, seperaherquamide, fumagilin, marcfortine, peniprequinolone, patulin, penicillic acid, and gliotoxin (Li and Zhang, 2013). In this

study, the four *Penicillium* isolates demonstrated nematicidal activity from crude extracts of secondary metabolites. Treatment with crude extracts of secondary metabolites was able to cause > 50% mortality of the nematode *P. coffeae*.

CONCLUSIONS

Five fungi isolate that have the potential as biocontrol agents of *P. coffeae* have been isolated from the rhizosphere of coffee plants. The antagonistic mechanisms of them against *P. coffeae* is through the production of protease enzymes and secondary metabolites. Isolates PIIIB0402b and PIVA0402a have the most potential compared to other isolates to be developed as biocontrol agents for parasitic plant nematodes. Based on the identification results using phenotypic characters and molecular analysis on the ITS rDNA region, the five selected isolates were identified as *Penicillium rubidurum, Penicillium steckii*, *Penicillium janthinellum,* and one isolate has not yet been identified at the genus level but is considered as Ascomycota.

ACKNOWLEDGMENTS

This study was funded by The Directorate of Research and Community Service, Directorate General of Research and Development Strengthening, The Ministry of Research, Technology, and Higher Education of Indonesia, which is greatly appreciated.

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Siwi Indarti	:	Soil sampling, research & methodology planning, drafting manuscript
Donny Widianto	:	Soil sampling, research & methodology planning, data interpretation, editing manuscript
Tri R. Nuringtyas	:	Evaluating & analyzing data interpretation
Nur A. Arofatullah	:	Data analysis, formatting & editing manuscript
Irfan D. Prijambada	:	Evaluating and analyzing data interpretation