JOURNAL OF TROPICAL LIFE SCIENCE
2020, Vol. 10, No. 1, 35 – 42
http://dx.doi.org/10.11594/jtls.10.01.05

Research Article

The Potency of *Trichoderma* sp. as A Biocontrol Agent against *Fusarium* sp. Pathogen of Porang (*Amorphophallus muelleri* Blume) Tuber

Nindi Nazula Fajarini, Rodiyati Azrianingsih *, Suharjono

Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang 65145, Indonesia

Article history:
Submission April 2019
Revised August 2019
Accepted November 2019

**Corresponding author:*
E-mail: rodiyati@ub.ac.id

ABSTRACT

Porang (*Amorphophallus muelleri* Blume) has high economic value in Asia. *Porang tuber* is high in glucomannan and thus becomes one of the exportable food commodities. *Porang* plants are susceptible to wilt disease caused by *Fusarium* sp. *Trichoderma* sp. has the ability to inhibit the growth of pathogenic fungi such as *Fusarium* sp. This research aimed to study the potency of *Trichoderma* sp. in controlling the growth of *Fusarium* sp. *Trichoderma* sp. was isolated from the rhizosphere soil in *A. muelleri* plantation, while *Fusarium* sp. was isolated from *A. muelleri* tuber infected by *Fusarium*. The fungi isolates were identified phylogenetically based on the similarity of Internal Transcribed Spacers (ITS) sequence. Both fungi were antagonistically assessed based on the dual culture method. The antagonistic assay showed that the two isolates of *Trichoderma* had the potency to inhibit the growth of the two isolates of *Fusarium*. *Trichoderma* sp. 2 has higher antagonistic potency than *Trichoderma* sp. 1. Based on ITS sequence similarity, *Trichoderma* sp. 1 and *Trichoderma* sp. 2 were identified as *Trichoderma longipale* and *Trichoderma spirale* respectively, while both pathogenic *Fusarium* were identified as *Fusarium oxysporum*. *Trichoderma spirale* therefore could be developed as a biopesticide agent in controlling *Fusarium oxysporum*.

Keywords: Amorphophallus oncophyllus, Antagonistic Fungi, Fungicide, ITS Sequence, Yellow Konjac

Introduction

Porang (*Amorphophallus muelleri* Blume) belongs to the Araceae Family and grows well as an agroforestry crop in Java [1]. This plant's tuber is known having high economic value in Asia [2] due to its up to 80% glucomannan content [3]. Therefore, it becomes one of the widely-exported commodities for food needs. The market demand for *Porang* is higher than its production. This is due to the improper postharvest handling that is potential to trigger the infection by the pathogenic microorganisms such as fungi. Several types of fungus that can infect tubers are *Phytophthora infestans* [3], *Fusarium oxysporum* [4], and *Botrytis cinerea* [5].

Fusarium species are the plant pathogens that are well-known as the cause of economic damage in agricultural productions [6]. They are economically harmful because most of the members are the causal agents of root rot in the agricultural crops around the world [7]. *Fusarium* wilt spread in many Asian countries such as India, Indonesia, Malaysia, Taiwan, and Philippines [8]. *Fusarium* species have been found to infect agricultural crops such as banana [9], strawberry [10], chili [11] and konjac [5].

Chemical compounds have been used to control plant diseases, but the overuse of the compounds has caused the development of pathogens resistant to fungicides [12]. The biocontrol agents have the ability to compete with pathogens for nutrients and to inhibit the pathogen's growth by secreting antibiotics or reducing the pathogen populations through parasitism [13] and activation of

How to cite:
Fajarini NN, Azrianingsih R, Suharjono (2020) The Potency of *Trichoderma* sp. as A Biocontrol Agent Against *Fusarium* sp. Pathogen of Porang (*Amorphophallus muelleri* Blume) Tuber. Journal of Tropical Life Science 10 (1): 37 – 42. doi: 10.11594/jtls.10.01.05

multiple mechanisms [14].

The rhizosphere is a part of the soil around the roots of plants and has a role as an external defense for plants against the attack of root pathogens. A population of microorganisms in the rhizosphere is usually more numerous and diverse than in the non-rhizosphere soil [15]. They can be beneficial to the plant since they suppress or harm the pathogens through the competition for nutrients [16].

The soil microbes such as *Trichoderma, Penicillium*, etc., can be some better alternatives since they reside in the rhizosphere of the crop plants and are effective to suppress the pathogens [17]. *Trichoderma* is easily isolated from soil, decaying wood, and other forms of plant organic matter. It is proven to have induced the programmed cell death in plant fungal pathogen such as *Fusarium* sp. [18].

Chemical fungicide is one of the methods to eradicate the plant diseases, but it is rarely found in the management of postharvest pathogens of *A. muelleri*. The previous study has identified the postharvest pathogens in *A. muelleri* without conducting any antagonistic assay [5]. Based on the potency of *Trichoderma* in suppressing the development of pathogenic fungi such as *Fusarium*, it is necessary to conduct a research to determine the antagonistic inhibition level on the growth of pathogenic fungi (*Fusarium*) found in *Porang* tubers.

Material and Methods

Isolation of fungal cultures

Two isolates of *Fusarium* sp. (F1 and F2) were isolated from the tubers of *A. muelleri* aged 6 weeks and 10 weeks after the harvest in Bantur Village, Malang Regency, East Java Province, Indonesia. The tuber's infected vascular tissue was cut with a sterile knife (*ca.* 1 × 1 × 0.2 cm^3). The samples were sterilized in 5.25% (w/v) of sodium hypochlorite solution for 30 seconds and were rinsed thrice with sterile water for 1 minute and then were placed on the surface of Potato Dextrose Agar (PDA) medium. The PDA medium contained 50 ppm *streptomycin* to minimize the chance of bacterial growth. Plates were incubated at 28 ± 2 °C and were observed periodically. The fungi were identified following the sporulation and a single colony was picked for purifying the culture and then was preserved at 0-4 °C on PDA slants [7].

Trichoderma isolates were obtained from the rhizospheric soil of *A. muelleri* crops in Bantur, Malang, East Java, Indonesia. The soil samples were collected from various sources at three locations of *A. muelleri* fields. The samples were put into a cool box container to maintain the viability of the fungi. For fungal isolation, 25 g of rhizospheric soil sample was suspended in 225 mL of 0.85% (w/v) sodium chloride solution and manually homogenized for a few minutes. 1 mL of suspension was diluted into 9 mL of 0.85% (w/v) sodium chloride solution in the test tube and then shaken until being homogeneous [19]. Afterwards, a serial dilution performed up to 10^{-5} dilutions [20]. 0.1 mL suspension of soil samples on each dilution was inoculated into the Petri dishes using the "poured plate" method with PDA medium containing 50 ppm of *streptomycin* to prevent bacterial growth. Cultures were incubated at 28 ± 2 °C for 5-7 days and the number of growing colonies were calculated [21].

Fungal morphological characterization

Each isolate of fungi was purified from a single spore. A single spore of fungal isolates was grown for 10-15 days on PDA medium containing 50 ppm of *streptomycin*. The macroscopic colony culture was characterized by having the color, shape, and texture of the colony. The microscopic features of hyphae and reproductive structure were characterized based on Ellis *et al*. [22].

Antagonist assay of Trichoderma isolates against Fusarium sp.

The antagonist assay corresponds to the dual culture method by Sibounnavong [23]. A single spore of *Trichoderma* isolate was grown together with *Fusarium* isolate spore in a PDA at a distance of 3 cm (Figure 1). The culture was incubated at 28 ± 2 °C for 7 days. As a control, *Fusarium* isolates spore is grown on the media without *Trichoderma* isolate. Each experiment was conducted with three replications. The control and the antagonist assay were observed on the 4^{th} day to the 7^{th} day of incubation. The ability of *Trichoderma* to inhibit the *Fusarium* growth can be calculated by the Percentage Inhibition of Radial Growth (PIRG) formula.

$$\boldsymbol{PIRG}\ (\%) = \frac{\boldsymbol{R1 - R2}}{\boldsymbol{R1}} \times \boldsymbol{100}\%$$

Note:
R1: diameter of pathogens colonies on control media (cm)
R2: diameter of pathogens colonies on dual culture media (cm)

Figure 1. Dual culture antagonism assay

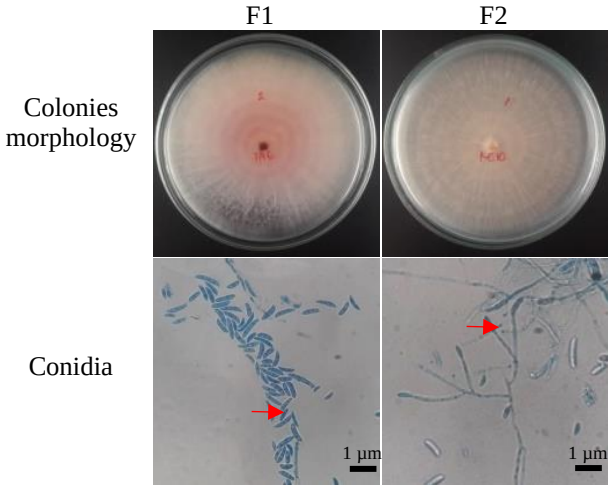


Figure 2. Morphological characteristic of *Fusarium* sp.

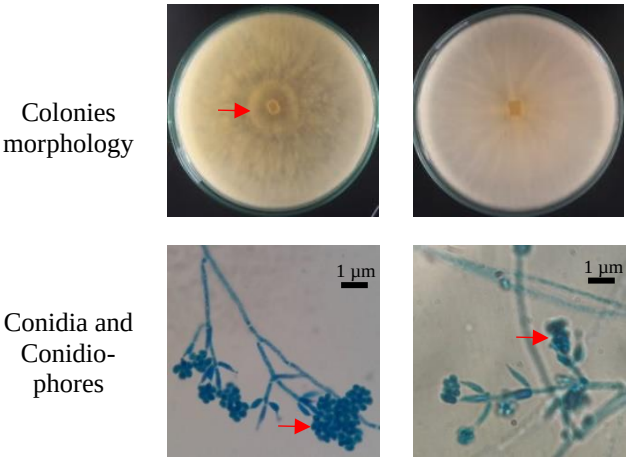


Figure 3. Morphological characteristic of *Trichoderma* sp.

Fungal identification base on ITS sequence

Phylogenetic identification of *Fusarium* and *Trichoderma* isolates was carried out based on the conserved ribosomal ITS region. Genomic DNA was extracted using the i-genomic Soil DNA Extraction Mini Kit Protocol (USA). The sequence of ITS regions was amplified using the universal primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [24].

The amplification of ITS region was performed on a *Thermal Cycler* with GoTaq® Green Master Mix, 2× 25 µL; 10 µM upstream primer 0.5–5.0 µL; 10 µM downstream primer 0.5–5.0 µL; < 250 ng DNA template 1–5 µL; Nuclease-Free Water to 50 µL N.A [25] with modifications. The DNA sequence amplification program of PCR machine consists of an initial denaturation at 95°C for 2 minutes followed by 35 cycles: denaturation at 94°C for 30 seconds, annealing 55°C for 1 minute, and extension 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The amplified PCR products were verified on an electrophoresis gel agarose (1.5% w/v) in 1X TAE buffer at 65 V for 150 minutes. The ITS sequence was purified and sequenced at First Base, Malaysia. The ITS nucleotide sequences for each isolate were compared to those in the public domain databases of the National Center for Biotechnology Information (NCBI); www.ncbi.nih.gov using the Basic Local Alignment Search Tool for Nucleotide Sequences (BLASTN). The alignment of ITS DNA sequences was done using the ClustalW program. The phylogenetic tree was constructed using the MEGA version 6 program with 1000 bootstrap, while the inversion was conducted using the Maximum Likelihood or Unweighted Pair Group Method for Arithmetic Analysis (UPGMA) [24].

Results and Discussion

Morphological characteristic of Fusarium isolate

The two *Fusarium* isolates were isolated. According to Ellis [22], *Fusarium* grows rapidly with pale-white or bright-colored colonies with or without air mycelium (Figure 2). Asexual reproduction in *Fusarium oxysporum* is accomplished by macroconidia, microconidia and chlamydospores [26]. Microconidia was found in the monofialid and oval or fusiform [27]. Macroconidia are produced

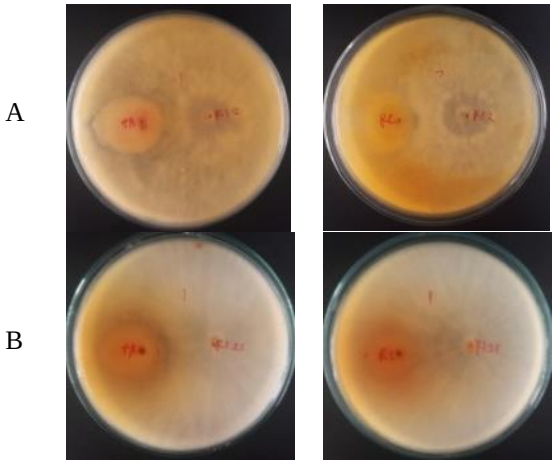


Figure 4. The inhibition of *Trichoderma* sp. against *Fusarium* sp., A. *Trichoderma* sp. (T1) against *Fusarium* F1 and F2 isolates, B. *Trichoderma* sp. (T2) against *Fusarium* F1 and F2 isolates

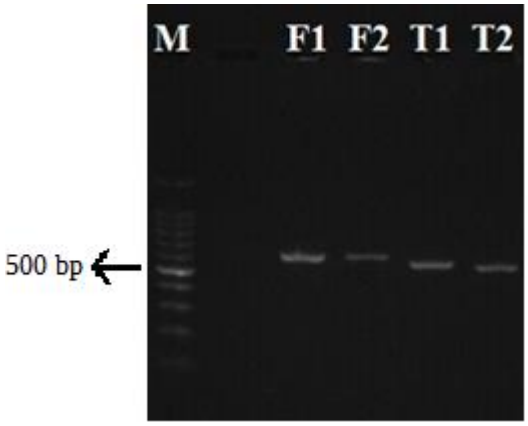


Figure 5. Amplified of ITS regions of *Fusarium* and *Trichoderma* isolates with primer ITS4/ ITS5; F1 and F2 are *Fusarium* isolates, T1 and T2 are Trichoderma isolates, while M is a marker.

abundantly, multinucleated, and germinate rapidly. It reproduces fungus efficiently. Macroconidia of *Fusarium* is straight or curved and thick [28]. Based on structures of microconidia, macroconidia, colony's pigments, and the other morphological characters, F1 was identified as *Fusarium* sp. (F1) and also F2 was identified as *Fusarium* sp. (F2).

Morphological characteristic of Trichoderma isolates

According to Ellis [22], *Trichoderma* sp. has yellowish colonies with surface textures like cotton. Usually, it has a small zone like a concentric ring (Figure 3, T1). Based on Rianti [30] *Trichoderma* sp. grows fast and has a cotton-like texture. *Trichoderma* sp. has round conidia and conidiophores that has a bulkhead and branches. The main branch of the conidiophores arises directly from the main armpit. The conidiophores branching extends along the conidiophores [31].

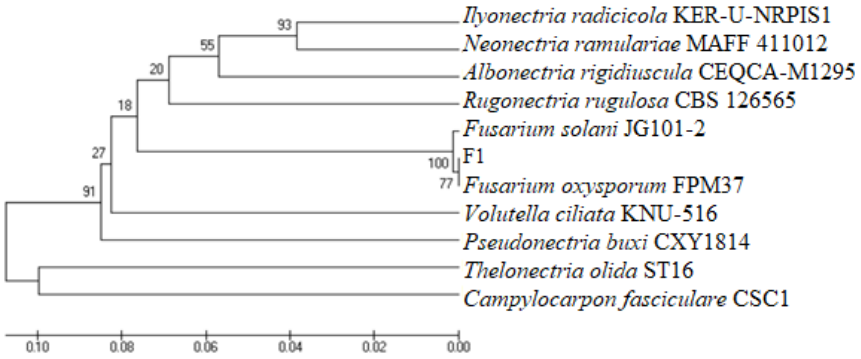
The potency of Trichoderma isolates to inhibit the growth of Fusarium isolates

As biocontrol agents, *Trichoderma* (T1 and T2) were assayed for their inhibition against the two *Fusarium* isolates (F1 and F2). Both T1 and T2 rhizosphere *Trichoderma* isolates have different potency in inhibiting both of the *Fusarium* isolates (Figure 7). The inhibition percentages of the T1 isolate against the *Fusarium* isolates F1 and F2 were 65.4% and 74.5% respectively. On the other hand, T2 isolate has the inhibition potency of 70.3 % and 79.8 % against the *Fusarium* F1 and F2 isolates respectively. Based on the previous result, *Trichoderma asperellum* was able to inhibit *Fusarium oxysporum* about 23-71% after six days incubation [28]. Moreover, *Trichoderma viride* showed that the inhibition of the mycelial growth of *Fusarium* sp. is 52.31% [32].

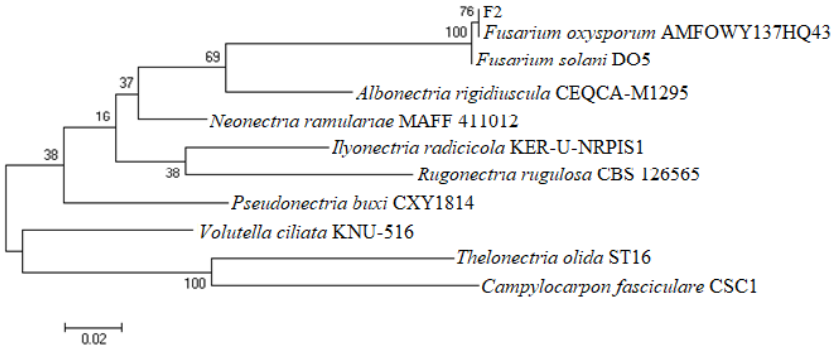
Antibiosis is an antagonistic interaction involving antibiotics that are produced by a microorganism which is detrimental to the growth of pathogens [14]. *Trichoderma* spp. produce antibiotics such as gliovirin, gliotoxin, viridin, viridol, koninginins, pyrones, and peptaibols to inhibit the fungal phytopathogens [33, 34].

The most important factor for fungi is substrate competition [35]. At the very common reason for the death of many microorganisms growing around of *Trichoderma* is the starvation due to limited nutrients and for rhizospheric colonization.

The process of mycoparasitism involves a direct attack of one fungal species on another one. According to Singh [14], mycoparasitism is one of the main mechanisms involved in the antagonisms of *Trichoderma*. The events leading to mycoparasitism are complex, taking place as follows: Chemotropic growth of *Trichoderma*, recognition of the host, coiling and appressoria formation, secretion of hydrolytic enzymes, penetrations of the hyphae, and lysis of the host.



(a)



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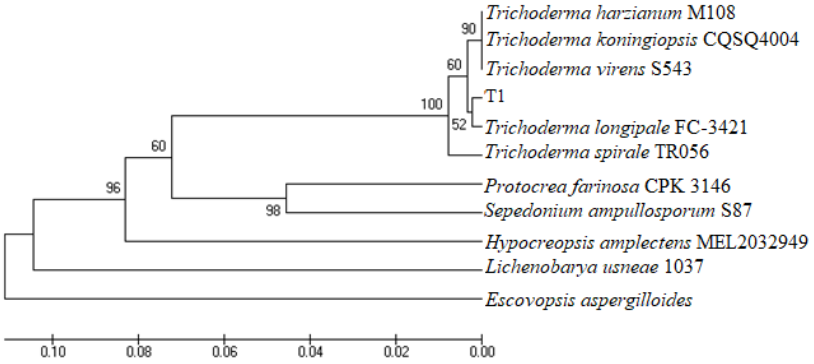
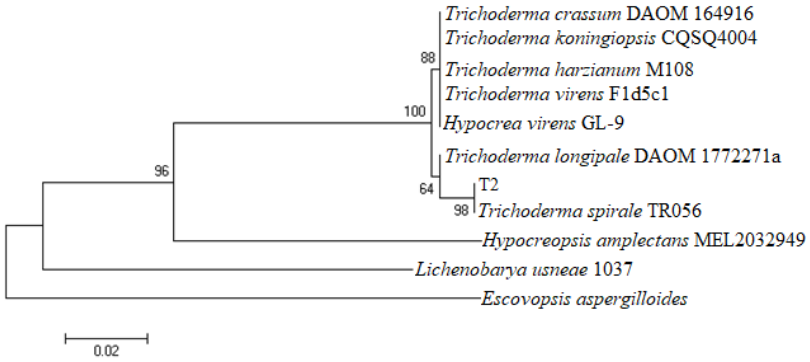


(c)



(d)

Figure 6. Phylogenetic tree generated using nucleotide sequence information of the ITS region by Maximum Likelihood (a and c) and UPGMA methods (b and d)

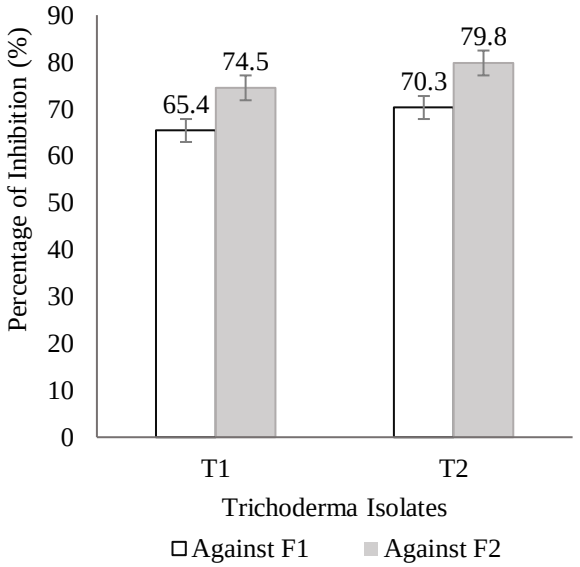


Figure 7. The potency of Trichoderma to inhibit the growth of Fusarium F1 and F2 on the 7th day dual culture assay

The possible inhibition mechanism of both T1 and T2 isolates was competition since the *Fusarium* isolates were not able to grow well because of the limitation of nutrition and space for growth (Figure 4).

Fungal species identified based on ITS

The total size of the ITS4 and ITS5 regions of the isolates varied from 500 to 600 bp (Figure 5). *Fusarium* sp. and *Trichoderma* sp. sequences obtained from the amplification of conserved ribosomal ITS region was compared with the sequences from the NCBI database using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/).

Sequence analysis of ITS

Phylogeny tree of *Fusarium* isolates (F1 and F2) and *Trichoderma* isolates (T1 and T2) was constructed based on the ITS sequence and was compared with the reference strains. ITS sequences of all isolates were aligned with the consensus region using the CLUSTAL W program with 1000 bootstrap replications. The topologies of the Maximum Likelihood and UPGMA trees were constructed using the MEGA version 6 program. The phylogenetic tree generated by the Maximum Likelihood explains that the probability of an evolutionary event at a certain point in the tree is stochastic modelled: below the maximum probability, the preferred phylogenetic tree is the tree with the highest probability. Besides, the UPGMA explains that the agglomerative hierarchical grouping is based on the average linkage method [36].

As shown in Figure 6, isolate F1 revealed a close phylogenetic relationship with *Fusarium* sp. F1 isolate had an identical sequence with *Fusarium oxysporum* FPM37 (100%) (Figure 6A). Meanwhile, F2 was identified as *Fusarium oxysporum* AMFOWY137HQ43 (Figure 6B). Based on the microscopic characterization, both F1 and F2 isolates produced macroconidia abundantly and were curved and thick. The T1 and T2 isolates had the highest relationship with *Trichoderma longipale* FC-3421 (Figure 6C) and *Trichoderma spirale* TR-056 (Figure 6D) with the similarity value of 99.5% and 100% respectively. Meanwhile, based on colonies morphology and microscopic characterization, both T1 and T2 isolates have different characteristic. *Trichoderma longipale* (T1) has a small zone like a concentric ring, while *Trichoderma spirale* (T2) does not. They have cotton-like texture, round conidia, branching conidiophores, and grow rapidly.

Conclusion

Fusarium sp. 1 (F1) and *Fusarium* sp. 2 (F2) isolates were found in *A. muelleri* tuber rot. *Trichoderma* sp. 1 (T1) and *Trichoderma* sp. 2 (T2) have the potency to inhibit the growth of both the *Fusarium* isolates. Isolate T2 has the highest potency as an antagonist for the tuber rot fungi.

Both *Fusarium* isolates were identified as *Fusarium oxysporum*, while T1 and T2 were identified as *Trichoderma longipale* and *Trichoderma spirale* respectively. *Trichoderma spirale* has the best potential to be developed as a biological control agent against the tuber rot fungi *F. oxysporum*.

Acknowledgment

The authors are thankful to the Brawijaya University for providing the laboratory facilities to carry out this present study, to Ministry of Research and Technology Republic of Indonesia for the financial support and to the Porang farmer in Bantur sub-district, Malang Regency for providing the valuable tubers.

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