ABSTRACT

Trichoderma spp. is a fungus widely used to control soil-borne pathogens, such as Rhizoctonia solani which is plant pathogenic fungi in widely host range, especially on rice. This research aimed to evaluate the ability of Trichoderma asperellum isolate UGM-LHAF against R. solani causing sheath blight disease of rice in vitro condition. Trichoderma sp. used in this research was obtained from The Biological Laboratory of Pakem, Yogyakarta, Indonesia, and Rhizoctonia sp. was obtained through isolation of diseased rice obtained from rice fields in Yogyakarta. The two isolates were characterized base on morphology and molecular identification based on ITS rDNA. The pathogenicity test of Rhizoctonia sp. was evaluated by adding four sclerotia of Rhizoctonia sp. near rice roots at 6 days after sowing. The in vitro test used dual culture and antifungal activity (0%, 10%, 25%, 50% culture filtrate of Trichoderma sp.) with three replicates of each treatment. Two isolates were identified as T. asperellum and R. solani. Sheath blight symptoms appeared after 12 days inoculation. In the in vitro test, T. asperellum isolate UGM-LHAF was able to inhibit the mycelial growth of R. solani (64.23% on dual culture and 68.5% on antifungal activity). This study suggests that T. asperellum isolate UGM-LHAF able to inhibit the growth of R. solani and can be a further potential candidate as a biocontrol agent against R. solani causing sheath blight disease of rice.

Keywords: Rhizoctonia solani; rice; sheath blight disease; Trichoderma asperellum

INTRODUCTION

Rice is an important staple food in Indonesia with the provinces of East, West, and Central Java producing the highest rates. From 2018 to 2020, the national rice production experienced fluctuations of 59.2, 54.6, and 55.2 million tonnes, respectively (Badan Pusat Statistik, 2020). According to Rabindran and Vidhyasekaran (1996), and Zhang et al. (2009), one of the factors that influence the fluctuation of rice production is plant disease, such as sheath blight disease caused by Rhizoctonia spp., with a yield loss of 2.5–50%. R. solani based on hyphal anastomosis was divided into 14 anastomosis groups (AG) namely AG1 to AG13, and bridging isolate (BI), while AG1 was divided into subgroups namely AG1-IA, AG1-IB, AG1-IC, and AG1-ID (Carling, 1996; Priyatmojo et al., 2001; Carling et al., 2002, Garcia et al., 2006). P. Singh et al. (2019) stated that this disease is difficult to control due to the pathogen’s high diversity, wide host, and ability to survive in the soil and plant residues by forming sclerotia.

Biological agents that have been reported to be effective against soil-borne pathogens were genus Trichoderma (Mukhopadhyay & Kumar, 2020). Trichoderma spp. such as T. virens, T. harzianum, and T. asperellum were known to be able to suppress sheath blight disease (Khan & Sinha, 2007; Naeimi et al., 2010; de França et al., 2015). T. asperellum was able to inhibit the growth of phytopathogenic fungi such as Fusarium camptocerus, F. oxysporum, F. solani, F. camptocerus, Alternaria alternata, Colletotrichum gloeosporioides, Ganoderma applanatum, Botrytis cinerea, and Cytospora chrysosperma (Yu et al., 2021).
in laboratories of the Indonesia Department of Agriculture to be used as biological agents in managing plant diseases (Dinas Perkebunan Provinsi Kalimatan Timur, 2017; Rismanto, 2020). One of them is *Trichoderma* sp. from The Biological Laboratory of Pakem which has not been identified by molecular identification. The study not only identifying *Trichoderma* sp. isolate from The Biological Laboratory of Pakem based on ITS ribosomal DNA, but it also evaluating its ability against *R. solani* causing sheath blight disease of rice under *in vitro* condition.

**MATERIALS AND METHODS**

The study was conducted at the Plant Disease Laboratory, Faculty of Agriculture, Universitas Gadjah Mada, Special Region of Yogyakarta, Indonesia from November 2019 until May 2020.

**Isolation, Morphological Identification, and Pathogenicity Test**

*Trichoderma* sp. used in this research was obtained from The Biological Laboratory of Pakem, Yogyakarta, Indonesia, then cultured on PDA and incubated at 28°C. *Rhizoctonia* sp. was isolated from diseased rice obtained of rice fields in Yogyakarta, Indonesia (7.8305 S; 110.3458 E). The leaf sheath sample was cut (2–3 mm), disinfected with 1.5% NaOCl for 1 minute, rinsed 3 times with sterile distilled water, dried on sterile filter paper, grown on PDA, and incubated for 2–3 days at a temperature of 28°C. Furthermore, fungal hyphae with the characteristics of *Rhizoctonia* sp. was cultured on a new PDA for further analysis (Wang et al., 2015). The morphological features of *Trichoderma* sp. were observed, such as colony (color, size, shape), phialides (size), conidia (size, color, shape), and the growth rate (Bissett, 1991). Meanwhile, the morphological features of *Rhizoctonia* sp. were observed, i.e. colony, sclerotium characteristics (color, shape, number, and size), hyphae color and structure, as well as growth rate (Guleria et al., 2007; Susheela & Reddy, 2013; Mishra et al., 2014).

The pathogenicity test of *Rhizoctonia* sp. isolate UGM-RBAF was carried out by adding sclerotia on the rice roots. Rice seeds of IR64 that have been soaked and incubated for 24 hours were planted in the planting medium in the form of a mixture of soil and sterile manure 4:1 (v/v) with a total of 5 kg. Each pot consisted of five rice seeds. Inoculation of *Rhizoctonia* sp. isolate UGM-RBAF was carried out by placing four sclerotia around the rice roots at 6 days after sowing. Rice plants covered with transparent plastic to keep moisture then observed until symptom appearance.

**DNA Extraction, Amplification, and Sequencing**

*Trichoderma* sp. isolate UGM-LHAF and *Rhizoctonia* sp. isolate UGM-RBAF (5 mm diameter, 5 days old) were grown separately in 50 ml PDB medium and shaken in a shaker (130 rpm) at 28°C for 5 days. The DNA was extracted from growing mycelium, according to the 2% CTAB method (Doyle & Doyle, 1990). ITS1 (5′-TCCGTAGGGTGAAC CTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGA TGC-3′) primer pairs were used to amplify the DNA with T100 Thermal Cycler (White et al., 1990).

PCR was performed in a total volume of 25 μl, which contained 12.5 μl PCR mix (Bioline®), 9.5 μl ddH2O, 1 μl forward primer, 1 μl reverse primer, and 1 μl genomic DNA. PCR amplification for *Trichoderma* sp. isolate UGM-LHAF was programmed with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 90 s, annealing at 55°C for 2 min, extension at 72°C for 3 min, and final extension at 72°C for 5 min (Jaisani & Pandey, 2017). PCR amplification for *Rhizoctonia* sp. isolate UGM-RBAF was programmed with an initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 15 s, extension at 72°C for 15 s, and final extension at 72°C for 7 min (Sandoval & Cumangun, 2019).

The PCR amplified products were resolved on 1% agarose gels and run at 100 volt for 25 min. The DNA bands were visualized with ethidium bromide under UV-illumination. Then the PCR products were sent to FirstBASE, Malaysia, for sequencing, with the sample gene sequence adjusted to the *Trichoderma* spp. and *Rhizoctonia* spp. gene sequences contained in the GenBank database via BLAST search at https://blast.ncbi.nlm.nih.gov/. ITS DNA sequences were aligned using Clustal W. Phylogenetic tree was constructed with Maximum Likelihood method by Mega-X programs (Tamura et al., 2011). Each isolate sequence was deposited in GenBank.
Dual Culture

Dual culture test was carried out by culturing *Trichoderma* sp. isolate UGM-LHAF and *Rhizoctonia* sp. isolate UGM-RBAF (5 mm diameter, 5 days old) on PDA with the opposite side to each other (Xian et al., 2019). Two isolates were placed 2 cm from the edge of the Petri dish. PDA plate with only *Rhizoctonia* sp. isolate UGM-RBAF was included as a control. Three replicates were maintained for each treatment and incubated at 28°C for 3 days. The encounter of hyphae between the two isolates was observed under a light microscope (Olympus CX21, Tokyo, Japan). Furthermore, the percentage of pathogen growth inhibition in each test was calculated based on Saravanakumar et al. (2016) with the formula:

$$ I = \left( \frac{C - T}{C} \right) \times 100 $$

where *I* denotes growth inhibition of the pathogen (%), *C* and *T* indicate radial growth of the pathogen in control and treatment.

Antifungal Activity Test

Antifungal activity test was carried out based on Meena et al. (2017), where a hyphal disc of *Trichoderma* sp. isolate UGM-LHAF (6 mm diameter, 5 days old) was cultured in 50 ml of PDB medium, shaken using a shaker (130 rpm), and incubated at 28°C for 15 days. The culture was filtered with Whatman paper No.1 and centrifuged (10,000 rpm) for 15 minutes. Furthermore, the supernatant obtained was filtered with a 0.22 μm millipore to obtain the culture filtrate. *Rhizoctonia* sp. isolate UGM-RBAF (5 mm diameter, 5 days old) were grown on PDA medium containing 0%, 10%, 25%, and 50% culture filtrate of *Trichoderma* sp. isolate UGM-LHAF. The control plate, PDA medium contained an equal volume of sterile water which was inoculated with *Rhizoctonia* sp. isolate UGM-RBAF. Each treatment included three replicates and incubated at 28°C for 2–3 days. The mycelial growth was measured and the percentage of pathogen growth inhibition in each treatment was calculated according to the dual culture test formula.

Scanning Electron Microscopy (SEM)

Hyphae of *Rhizoctonia* sp. isolate UGM-RBAF growing on the culture filtrate of *Trichoderma* sp. isolate UGM-LHAF were observed under a scanning electron microscope (SEM). Observation using SEM was carried out by preparing the sample for one night in 0.05 M phosphate buffer (pH 7.3) containing 4% glutaraldehyde. The samples were washed 3 times (for 15 minutes each) with phosphate buffer the next day. Sample dehydration was carried out with stratified ethanol series (30, 50, 70, 80, 90, and 100%) for 15 minutes then dried with CO₂ for 5 minutes and immediately placed on aluminum pieces. Samples were placed on a 400 mesh grid. The sample was painted by negative painting used ammonium molybdate to bind the transmitted electron emission so that there was a color contrast. Then the samples were observed under SEM (Pandian et al., 2016).

Statistical Analysis

The data were analyzed with one-way ANOVA and followed by Duncan’s Multiple Range Test at a significant level of 0.05 using DSASTAT 1.101 program.

RESULTS AND DISCUSSION

*Trichoderma* sp. isolate UGM-LHAF had greenish-white colony color that turns dark green when approaching old age, hyaline hyphae with a septum, and pyramidal conidiophore with the phialide branches, the phialide size (diameter 10.31 × 3.29 μm), conidia (green and globose-shaped, diameter 3.74 × 3.25 μm), and growth rate of 34.43 mm/day (Figure 1a-c). The characteristics of *T. asperellum* isolate UGM-LHAF were similar to *T. asperellum* characterized by Shang et al. (2020). Oszako et al. (2020) stated that *T. asperellum* had conidiophores to which branched phialide, 2–3 in number, measuring 6.4 × 1.4 μm. Conidia were colored greenish, ellipsoid, 3.1 × 2.6 μm, and chlamydospores unicellular, terminal, and roughly spherical measuring 20.5 × 21.2 μm.

*Rhizoctonia* sp. isolate UGM-RBAF had perpendicular hyphae with a septum, a white colony that change to brownish-white when old, light brown to dark brown sclerotium with a round shape (diameter of 1.33–1.58 mm, the weight of 0.17 g/Petri dish, and amount of 19.5/petri), and a growth rate of 40 mm/day (Figure 1d-f). The characteristics of *R. solani* isolate UGM-RBAF were similar to *R. solani* characterized by R. Singh et al. (2015). Budiarti et al. (2020) reported that 41 *R. solani* isolates isolated
from Indonesia had cultural and morphological characteristics, i.e. hyphae branching of \( R. \text{solani} \) at 90 angles, had hyphal width (5.66–7.58 µm), a sparse fluffy of colony texture, sclerotia dark brown color, the irregular distribution pattern of sclerotia, micro, and macro sclerotial size (3.33 to 183.67 mm), and sclerotia on the surface of PDA. The pathogenicity test of \( Rhizoctonia \) sp. isolate UGM-RBAF explained that the first sheath blight symptom was observed at 12 days after inoculation. The mycelium was observed after 18 days inoculation around sheath blight symptoms (Figure 2a-b), while sclerotia were observed after 30 days inoculation (Figure 2c). This study indicated that \( Rhizoctonia \) sp. isolate UGM-RBAF was a virulent pathogen on rice.

The morphological identification of \( Trichoderma \) sp. isolate UGM-LHAF and \( Rhizoctonia \) sp. isolate UGM-RBAF were confirmed with molecular identification using ITS1–ITS4 Primers. The results showed that the possibility of amplifying PCR products with sizes around 600 bp for \( Trichoderma \) sp. isolate UGM-LHAF (Figure 3a), and around 700 bp for \( Rhizoctonia \) sp. isolate UGM-RBAF (Figure 3b). The result for the analysis of the two sequences using BLAST showed that \( Trichoderma \) sp. isolate UGM-LHAF was identified as \( Trichoderma \) asperellum (MT102403) with 100% identity, while \( Rhizoctonia \) sp. isolate UGM-RBAF was identified as \( Rhizoctonia \) solani (MN365714) with 96.5% identity. It was strongly confirmed their morphological characteristic. The two sequences were then deposited at NCBI with the accession number MT367901 (\( Trichoderma \) asperellum isolate UGM-LHAF) and MT367900 (\( Rhizoctonia \) solani isolate UGM-RBAF).

The phylogenetic analysis based on the ITS sequence observed that \( T. \) asperellum isolate UGM-LHAF was located in the clade \( T. \) asperellum strain MF22552, PANCOM8 isolate, and Tasum66 isolate with 100% bootstrap value (Figure 3a). \( T. \) asperellum strain MF22552 was isolated from lakes in India, \( T. \) asperellum isolate PANCOM8 isolated from soil in Indian rice fields, and \( T. \) asperellum isolate Tasum66 isolated from soil in China. The phylogenetic tree based on the ITS sequence (Figure 3b) showed that \( R. \) solani isolate UGM-RBAF was grouped with \( Thanatephorus cucumeris \) isolate JZ2, \( R. \) solani strain APHyd,
Figure 2. Pathogenicity test of *Rhizoctonia* sp. isolate UGM-RBAF. (a-b) symptoms of sheath blight disease (white arrow) 18 days after inoculation on IR64 rice. (c) sclerotia (red arrow) was observed on 30 days after inoculation.

Figure 3. Molecular identification and phylogenetic tree analysis. (a) M = 1 Kb ladder, lane 1 = *Trichoderma asperellum* isolate UGM-LHAF; (b) M = 100 bp ladder, lane 1 = *Rhizoctonia solani* isolate UGM-RBAF. Bootstrap values with 1000 replicates are used in phylogenetic analysis. Isolates from this study are in red node. *Fusarium falciforme* is an outgroup.
R. solani isolate RS7 KANCHIPURAM by 96% bootstrap value. R. solani isolate RS7 KANCHIPURAM was the result of isolation from symptoms of sheath blight on rice leaves in India, and Thanatephorus cucumeris isolate JZ2 was also the result of isolation from rice plants in China. V. Singh et al. (2018) reported that R. solani APHyd strains were obtained from the isolation of symptoms of sheath blight on maize in India which were grouped into AG1-IA based on ITS sequences. This showed that R. solani isolate UGM-RBAF belongs to the AG1-IA subgroup.

The dual culture test showed that hyphal of T. asperellum isolate UGM-LHAF after one day incubation was contacted with hyphal R. solani isolate UGM-RBAF (Figure 4a-b). The colony diameter of R. solani isolate UGM-RBAF after 3 days incubation had 23.5 mm (in dual culture Petri dish) and 65.7 mm (control Petri dish). T. asperellum isolate UGM-LHAF was able to suppress the radial growth of R. solani isolate UGM-RBAF. The inhibition of T. asperellum isolate UGM-LHAF against R. solani isolate UGM-RBAF in mycelial growth was found as 64.23%. The effectiveness of T. asperellum isolate UGM-LHAF in controlling R. solani in vitro by a dual culture test in this study was in line with Asad et al. (2014) and Chinnaswami et al. (2021) studies, which stated that T. asperellum was able to suppress the radial growth of R. solani at a percentage range of 67.74–74.40%. The inhibition zone of the two isolates illustrated the coiling of T. asperellum isolate UGM-LHAF hyphae against R. solani hyphae (Figure 4c). In line with Jiang et al. (2016) reported that T. asperellum coiled and penetrated the hyphae pathogen then breaks it down into smaller fragments so that causes the death of pathogenic hyphae.
According to Wu et al. (2017), *T. asperellum* had a mycoparasitic mechanism in the form of hyphae coiling involving cell wall-degrading enzymes (CWDEs) comprising of chitinase, glucanase, and protease which degraded pathogenic cell walls. *Trichoderma* hyphae coiled pathogenic hyphae and caused hyphal abnormalities and pathogenic cell lysis (Romero-Cortes et al., 2019; Zhang & Zhuang, 2020).

The culture filtrate inhibitor of *T. asperellum* isolate UGM-LHAF also had significant differences with control in reducing the mycelium growth of *R. solani*. Table 1 showed that the radial growth of *R. solani* mycelium at 10%, 25%, and 50% concentration of culture filtrate of *T. asperellum* isolate UGM-LHAF was 79.22 mm, 69.35 mm, 28.35 mm, respectively. The percentage of growth inhibition of *R. solani* after 2 days incubation in each treatment were 11.98%, 22.94%, and 68.50%, respectively. Mayo-Prieto et al. (2020) reported that *Trichoderma* sp. and *Rhizoctonia* sp. were identified as *T. asperellum* and *R. solani* based on ITS ribosomal DNA. *In vitro* test showed that *T. asperellum* isolate UGM-LHAF was able to inhibit the growth of *R. solani* on dual culture and antifungal test.

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**CONCLUSION**

*Trichoderma* sp. and *Rhizoctonia* sp. were identified as *T. asperellum* and *R. solani* based on ITS ribosomal DNA. *In vitro* test showed that *T. asperellum* isolate UGM-LHAF was able to inhibit the growth of *R. solani* on dual culture and antifungal test.

**ACKNOWLEDGEMENT**

This research article is part of the first author’s thesis. The authors would like to thank The Indonesian Endowment Fund for Education (LPDP)—Ministry of Finance, Indonesia, for giving a National-International Olympic Affirmation Scholarship and funding this research.

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**Table 1. The inhibition effect of culture filtrate of *Trichoderma asperellum* isolate UGM-LHAF toward mycelial growth of *Rhizoctonia solani* isolate UGM-RBAF at 2 days**

| Concentration | Radial growth (mm) | Inhibitory effect (%) |
|---------------|--------------------|-----------------------|
| 0% (control)  | 90.00              | 0 d                   |
| 10%           | 79.22              | 11.98 c               |
| 25%           | 69.35              | 22.94 b               |
| 50%           | 28.35              | 68.50 a               |

1 The radial growth of *Rhizoctonia solani* isolate UGM-RBAF was measured on four sides in each treatment plate.
2 The numbers followed by the same letter in the same column show no significant difference in the 5% DMRT test.
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ISSN 1410-1637 (print), ISSN 2548-4788 (online)