

# Screening of endophytic fungi as potential antagonistic agents of *Pyricularia oryzae* and evaluation of their ability in producing hydrolytic enzymes

NOVIA DWI PUTRI\*, LILIEK SULISTYOWATI, LUQMAN QURATA AINI, ANTON MUHIBUDDIN, IRISA TRIANTI

Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia.

Tel./Fax: +62-341-575843, \*email: novia.dwi.putri26@gmail.com

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**Abstract.** Putri ND, Sulistyowati L, Aini LQ, Muhibuddin A, Trianti I. 2022. Screening of endophytic fungi as potential antagonistic agents of *Pyricularia oryzae* and evaluation of their ability in producing hydrolytic enzymes. *Biodiversitas* 23: 1048-1057. One of the most important phytopathogenic fungi is *Pyricularia oryzae*, as it is the causative agent of rice blast diseases, which is the most destructive and detrimental disease in rice. Biological control using endophytic fungi can be an alternative to control blast diseases. Endophytic fungi are fungi that colonize internal plant tissues without apparently damaging the host. The aim of this study was to isolate endophytic fungi that produce hydrolytic enzymes and to investigate their ability to suppress blast disease. In this study, fifty strains of endophytic fungi were successfully obtained from rice leaves. Three fungi with the highest inhibition against *P. oryzae* were identified as *Trichoderma asperellum*, *Curvularia chiangmaiensis*, and *Fusarium solani* by analyzing fungal ITS sequence. They can produce chitinase ranging from 1.43 to 151 µg/mL and cellulase ranging from 1.83 to 4.09 µg/mL, which were hydrolytic enzymes that account for the lysis of phytopathogens. This enzymatic activity can cause damage and degradation of cell walls. SEM results revealed that these endophytic fungi probably excreted wall lytic enzymes or antifungal substances, inflicting wrinkles and disintegrating *P. oryzae* mycelium.

**Keywords:** Cellulase, chitinase, *Curvularia chiangmaiensis*, *Fusarium solani*, rice blast, *Trichoderma asperellum*

## INTRODUCTION

*Pyricularia oryzae* Cavara (teleomorph: *Magnaporthe oryzae*) is one of the most important phytopathogenic fungi because it is the causative agent of rice blast diseases, the most destructive and detrimental disease in rice (Gabriel et al. 2022). *Pyricularia oryzae* affects more than 50 species of grasses such as wheat, barley, oats, and millet (Dean et al. 2012; Langner et al. 2018; Kurrata et al. 2019). Outbreaks of rice blast diseases are a steady hazard to cereal manufacturing worldwide and may bring about an annual lack of rice enough to feed more than 60 million people (Choi et al. 2013). Rice blast disease in panicles and leaves of the plant can cause a loss in grain yield ranging from 11.90% to 37.80%; at the same time, tiller and seed losses because of this disease range from 20-78.19% and 7.97-64.48% (Chuwa et al. 2015). The pathogenic fungus *P. oryzae* can attack rice plants at various stages. In the vegetative stage, the pathogen can infect leaves (leaf blast), while in the generative stage, the pathogen can infect the leaves and panicles (panicle blast) (Liu et al. 2016). The pathogen is mainly spread throughout the year/growing season by air. Blast pathogens can survive on field-infected rice scraps. Spore formation of the fungus has been observed on stems left in mulch for up to 18 months (Raveloson et al. 2018). In the early stage of infection, invasive hyphae invade the host cell, which leads to the development of the first symptoms in the form of oval spots (Giraldo and Valent 2013; Zhang et al. 2014).

The most common approach to combat blast disease is to use resistant strains of rice plants. Initially, the use of resistant strains was effective in controlling blast disease. However, in most cases, host resistance becomes ineffective due to the emergence of recent blast races (Kurrata et al. 2019). High genetic variation or genomic adaptation is one of the mechanisms of *P. oryzae* that can overcome host resistance to prevent host recognition (Longya et al. 2020). In this regard, biological control could be an effective alternative to control blast disease. Biocontrol of diseases in plants controls the population of phytopathogens with the aid of living organisms (Heimpel and Mills 2017; O'Brien 2017). Biological control agents are commonly obtained from fungal or bacterial strains that are isolated from the endosphere, phyllosphere, or rhizosphere (O'Brien 2017). The mode of action by biocontrol agent microbes towards plant pathogens is competition, parasitism, antibiosis, and induced resistance. Antagonists that act by hyperparasitism or antibiosis directly interfere with pathogens. Such interactions constitute an extremely controlled series of metabolic events, often combining other mechanisms of action (Kohl et al. 2019). Especially as hyperparasites, biocontrol agents penetrate and kill the hyphae, spores, and dormant structures of plant pathogens such as fungi, bacteria, or others (Ghorbanpour et al. 2018). For antibiosis action, microbial biocontrol agents produce specific or non-specific metabolites with antifungal or antibacterial activity. Several studies have characterized the

antibiotic substances produced by biocontrol agents (Kohl et al. 2019).

Endophytic fungi colonize internal plant tissues without inflicting obvious damage to their host (Kumar et al. 2017; Tadych and White 2019). Endophytic fungi have been reported to be capable of producing hydrolytic enzymes such as chitinase (Kumar et al. 2018) and cellulase (Robl et al. 2013). Chitinase and cellulase are responsible for lysing cell walls of fungal phytopathogens. As a result, endophytic fungi play a vital role in the biological control of numerous plant pathogenic fungi by inhibiting the mycelial growth of the pathogens (Jadhav et al. 2017; Thambugala et al. 2020). Therefore, the aim of this research was to screening of endophytic fungi based on their ability to inhibit the mycelial growth of *P. oryzae* and to investigate their capability to produce hydrolytic enzymes.

## MATERIALS AND METHODS

### Isolation, identification, and pathogenicity test of *Pyricularia oryzae*

*Pyricularia oryzae* was isolated using a single spore isolation technique (Li-wang et al. 2019). Symptomatic leaves were cut and then inoculated in water agar (WA) medium. The leaves were taken out from 1.5% WA medium, and then the petri dish containing fungal spores was observed under a stereo microscope using Olympus Stereomicroscope System SZX7. Then fungal spores were taken with the help of dissecting needles. The isolated spores were then transferred to oatmeal agar (OMA) media (50 g/L oatmeal, 15 g/L agar) and incubated at 25°C for 5 days. The fungus was identified to the genus level with the aid of observing characteristics of aerial mycelium, reverse colony, conidia, conidiophores, and hyphae Barnett and Hunter (1998) and Klaubauf et al. (2014).

The pathogenicity test was carried out by inoculating *P. oryzae* isolates into the leaves of healthy rice plants. Fungal colonies were harvested using a brush by adding 10 mL of sterile distilled water (dH<sub>2</sub>O), including 0.02% Tween 20. The *P. oryzae* inoculum was sprayed on rice plants aged 18-21 days after planting (Kurrata et al. 2019). The development of lesions and length were also measured.

### Exploration and morphological identification of endophytic fungi

In this study, the sampling of rice plants was carried out in rice fields located in Karangploso, Malang, Indonesia. Endophytic fungi were isolated from healthy leaves, stem, and root pieces of rice plants, according to Khalil et al. (2021), with some modification. Each part of rice plant was rinsed thoroughly with running tap water to eliminate dust, soil particles, and debris and each part was then cut to approximately 1 cm pieces. All parts were initially subjected to surface sterilization with 70% alcohol and 1% sodium hypochlorite (NaOCl), each for 1 minute. Thereafter, the plant parts were rinsed two times with sterile dH<sub>2</sub>O for 1 minute, then dried on sterilized wipes. Next, the samples were inoculated in a Petri dish (9 cm

diameter) containing potato dextrose agar (PDA) media (consisting of 200 g/L potato infusion, 20 g/L agar, 20 g/L dextrose, and 0.1 g/L chloramphenicol) and incubated at 25°C for 10 days. The usage of chloramphenicol was recommended by Beuchat (1979) for routine laboratory analysis to suppress bacterial growth. The final rinse of the tissue segments was suppressed on the surface of the PDA media to verify the effectiveness of the surface sterilization process and to ensure that the fungus was isolated only from the insides of internal plant tissues. The lack of fungal growth on the PDA confirmed that the surface sterilization process surely removed fungi from the surface. Colony characteristics of the isolated endophytic fungi were determined from pure cultures grown on PDA. After the culture plates were incubated at 25°C for 7 days, the isolates were identified based on their colony color, surface texture, aerial hyphae color and shape reversal. Microscopic characteristics were observed and described based on color, shape, septation, measurement of conidia and conidiophores under a light microscope using Olympus CX33.

### Molecular identification of pathogenic and endophytic fungi

For molecular identification, mycelium of *P. oryzae* was inoculated into 2YEG medium (consisting of 2 g/L yeast extract and 10 g/L glucose) in an erlenmeyer flask and incubated at 100 rpm for 3 days at 27°C. Mycelia were then collected on filter paper according to Sornkom et al. (2015), while endophytic fungi that had the highest inhibitory ability in the antagonist test were grown on potato dextrose broth (PDB) media (containing the same composition as PDA without the agar). Then, the suspension was incubated for 3 days at 27°C. Molecular identification was carried out in several stages: isolation of fungal DNA, DNA amplification, and sequencing.

#### DNA extraction

Isolation of fungal DNA was performed according to the method of Sarkanj et al. (2018). Fungal mycelium that was grown on liquid media was collected on a mortar. Liquid nitrogen was added into the mortar and DNA was isolated using STES buffer method. 500 µL of STES buffer was added into 100 mg mycelia and incubated for 10 minutes at 25°C. Then, 150 µL of 3M potassium acetate was added and homogenized. Samples were centrifuged at 10,000 g for 1 minute. The supernatant was transferred to a new tube, and isopropanol was added according to the volume of the supernatant before being homogenized by turning it back and forth. Next, the sample was centrifuged at 10,000 g for 2 minutes. The supernatant was removed and 300 µL of 70% EtOH was added into pellet. This pellet was then centrifuged at 10,000 g for 1 minute; then, the EtOH was removed. The collected pellet was dried for 60 minutes at 25°C, and 100 µL of TE buffer was then added before being stored at -20°C.

#### DNA amplification

Amplification of the fungal DNA genome was amplified using universal primers ITS1 (5'-TCT GTA GGT

GAA CCT GCG G3 ') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990). The utilized Polymerase Chain Reaction (PCR) cycle involved 3 minutes of pre-denaturation at 95°C, 30 seconds of denaturation at 95°C, 30 seconds of annealing at 50°C, 1 minute of extension at 72°C, 1 minute of final extension at 72°C. The transition from denaturation to extension as a cycle was repeated 30 times. All PCR reactions were performed on a thermal cycler (MJ-Research Inc, Minicycler, PTC-150). The PCR product was analyzed by electrophoresis (Biorad, PowerPac basic Power Supply) in 1% agarose gel and given 0.75 µl of DNA stain. Gel and mold were immersed in 0.5x TBE buffer in the electrophoresis chamber (Biolab Major mini large horizontal gel electrophoresis tank). The utilized amounts of DNA Ladder marker and sample solution were 3 µL each. The bands were visualized in a UV Transilluminator (Dual Intensity Transilluminator, UVP TM-36).

#### Sequencing and DNA sequencing analysis

The PCR product was sequenced by PT. Genetica Science Indonesia (Tangerang, Indonesia). The DNA sequences of ITS region 1 and 4 were decided through the usage of BioEdit Sequence Alignment Editor, version 7.2.5. The sequences were compared to those in the database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/BLAST>).

#### *In vitro* Pyricularia oryzae growth inhibition assays

Inhibition growth of *P. oryzae* by endophytic fungi utilized the direct opposition method as described by Dhingra and Sinclair (1995). Endophytic fungi and pathogenic fungi were grown on PDA media. A 5 mm agar plug from the edge of the actively growing colony of endophyte and pathogen was positioned approximately 3 cm opposite each other and incubated at 28°C for 7 days. The *in vitro* antagonism of various isolates of endophytic fungi was determined by calculating the percentage of pathogen growth inhibition in the presence of endophytic fungi by the following formula:

$$PI = \frac{C - T}{C} \times 100\%$$

Where:

PI = Percentage of inhibition

C = Diameter of pathogen colonies in control treatment (cm)

T = Diameter of pathogen colonies in antagonist treatment (cm)

#### Scanning electron microscope (SEM)

The antagonist mechanism of endophytic fungi against the pathogenic fungus *P. oryzae* was observed with SEM. The sample that was taken depended on the result of the antagonist test that showed the highest inhibition of pathogens. Each antagonist mycelium was planted at one end of the agar plug and the pathogen at a distance of 3 cm. Small pieces of agar (approximately 2 mm<sup>2</sup>) were taken from the dual-cultures at the interaction zone, while the two

fungi had been at their early ranges of interaction. The pieces were coated with platinum and observed in a Hitachi TM3000 SEM at the Biosains Institute.

#### Quantification of hydrolytic enzymes produced by endophytic fungi

##### *Chitinase quantification*

The quantification of chitinolytic activity by endophytic fungi was performed according to the method of Agrawal and Kotasthane (2012) and Ferrari et al. (2014) with some modification. This was determined by the dinitrosalicylic acid (DNS) method, with colloidal chitin as a substrate. Quantitative observation was carried out by growing fungal isolates on liquid chitin media (consisting of 0.3 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 3 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>, 1 g citric acid monohydrate, 15 g agar, 3 g colloidal chitin) and incubated in a shaker at 200 rpm for 5 days at 28°C. Afterwards, the cultural filtrates were centrifuged at 3,000 rpm for 3 minutes. A 1 mL of supernatant was taken, to which was given 1 mL of 0.5% colloidal chitin in a phosphate saline buffer solution (0.1 M) before being incubated at 37°C for 15 minutes. Then 2 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added into solution and re-incubated at 100°C for 5 minutes. The absorbance of chitinolytic activity value was measured at 540 nm with a UH5300, Hitachi Inch spectrophotometer. A calibration curve with N-acetyl-β-glucosamine (NAG) was utilized as a standard to determine the reduction of saccharide concentration. Chitinolytic activity was measured by determining one unit (U) of the enzymes as the amount of enzyme needed to release 1 mmol of NAG in one hour at 37°C.

##### *Cellulase quantification*

For quantification of cellulase, endophytic fungi were cultured on yeast extract peptone medium supplemented with 0.1% of carboxymethyl cellulose (CMC) as a carbon source. Fungal isolates were grown on liquid peptone yeast media and incubated in a shaker at 100 rpm for 5 days at 28°C. After five days, suspensions were centrifuged at 3,000 rpm for 1 minute. The filtrate from liquid culture was utilized as a crude extract. The CMCase activity was determined according to the DNS method described by Potprommanee et al. (2017) by measuring the amount of reducing sugar liberated from CMC. 0.5 mL of supernatant was taken, then add 0.5 mL of 1% CMC that was dissolved in 0.1M phosphate buffer before being incubated at 50°C for 20 minutes. The reaction was stopped by adding 2.5 mL of DNS reagent. All mixtures were heated in boiling water at 100°C for 5 minutes to develop color. The absorbance values was measured at 575 nm with spectrophotometer. One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per minute.

#### Statistical analysis

Observational data of the pathogen growth inhibition assays, as well as chitinase and cellulase quantification, were analyzed with one-way analysis of variance (ANOVA). If the results were significantly different among the treatments, a post-hoc test was carried out with the

Duncan test at an error rate of 5%. Data were analyzed statistically with the SPSS program, version 22.0.

## RESULTS AND DISCUSSION

### Exploration and morphological identification of *Pyricularia oryzae*

Fungus was isolated from rice leaves, showing blast disease symptoms, as whitish or greenish-gray spots with dark greenish edges. As the infection progressed, the lesions gradually became whitish-greenish in color with a brownish-reddish necrotic margin (Figure 1.A). Result of morphological identification showed that fungus that grew on OMA was grayish in color and circular shape, with irregular margins (Figure 1.B). The mycelium covered a 9 cm diameter petri dish after  $\pm 14$  days. The mycelium grew to fill the petri dish measuring 9 cm in diameter after  $\pm 14$  days. Under the microscope, conidiophores were erect, straight or curved, unbranched, medium brown, and septate (Figure 1.C). The conidia were pyriform with tapered end, with middle cells being broader than other cells. The conidia had 2-3 septa and measured  $18.5\text{--}21.1 \times 6\text{--}6.7 \mu\text{m}$ . Pathogenic isolates that showed virulence on rice leaves were selected for further testing depending on the lesions of rice leaves on the surface. Symptoms of the rice blast appeared as gold-brownish lesions, while at the same time, the spots were rhombus or spindle-shaped.

### Fungal antagonist assay

A total of 50 endophytic fungal isolates were obtained and then evaluated for their ability to inhibit the mycelial growth of *P. oryzae*. The mechanism of antagonistic endophytic fungi against the *P. oryzae* was also observed. The result of the antagonist assay was indicated as percentage of growth inhibition of *P. oryzae* by endophytic fungi, which can be seen in Table 1 ( $F = 11.53$ ,  $P < 0.001$ ).

Three endophytic fungal isolates, namely SP3, D11, and D8, which had the highest antagonistic ability against *P. oryzae*, were selected to be tested for morphological and molecular observations, observation of the inhibitory mechanism by SEM, and production of chitinase and cellulase enzymes. In the fungal antagonist assay, isolate SP3 showed that the inhibition mechanism was mycoparasitism. This can be seen from the growth of SP3 mycelium on pathogenic fungi (Figure 2.A). Interaction of these two fungi was observed for several days, and it was seen that the mycelium of SP3 could be in direct contact after 3 days of observation. The treatment of the D11 fungus showed an antibiosis inhibitory mechanism (Figure 2.B). This indicates the presence of a clear zone between the D11 and *P. oryzae* fungi. Furthermore, isolate D8 also showed an antagonistic mechanism in the form of mycoparasitism and antibiosis (Figure 2.C).

### Molecular identification of pathogenic and endophytic fungi

The highest inhibition of *P. oryzae* was shown by SP3, D11, and D8 isolates and was then identified at molecular level. The sequences data were aligned using basic local

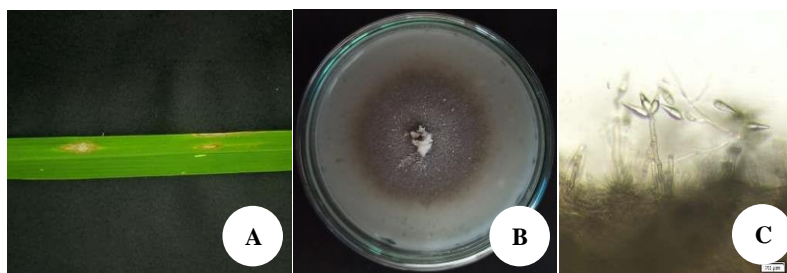
alignment search tool (BLAST). The pathogenic fungi were found to be the closest homologous of *P. oryzae* isolate PMB2 and the endophytic fungi were found to be the closest homologs of *T. asperellum* isolate Tasum66, *Curvularia chiangmaiensis* strain LC12044, and *Fusarium solani* isolate HJ12 (Table 2).

**Table 1.** Percentage of inhibition of *Pyricularia oryzae* by endophytic fungi

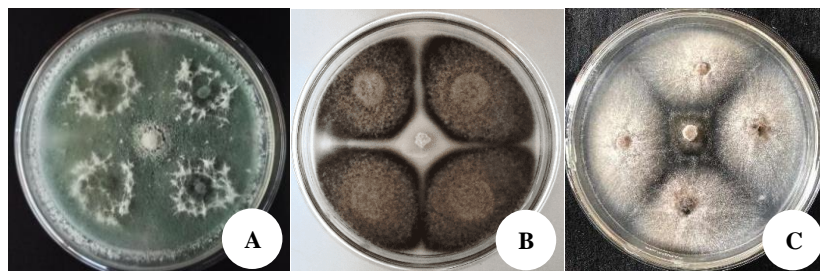
Endophytic fungi	Percentage of inhibition (%)
AA	61.62 $\pm$ 2.49 defgh
AB	67.68 $\pm$ 2.13 bcdef
AC	63.64 $\pm$ 1.19 cdef
AD	67.68 $\pm$ 3.46 bcdef
AE	67.68 $\pm$ 3.46 bcdef
ATR3	71.72 $\pm$ 3.30 abcd
AU	71.72 $\pm$ 1.74 abcd
BM	60.61 $\pm$ 5.44 efgh
D3	37.37 $\pm$ 2.27 j
D5	44.44 $\pm$ 0.64 ij
D6	43.43 $\pm$ 8.43 ij
D8*	73.74 $\pm$ 1.78 abc
D10	46.46 $\pm$ 1.83 ij
D11*	74.75 $\pm$ 0.30 ab
D13	44.44 $\pm$ 1.18 ij
DM	58.59 $\pm$ 1.35 fgh
EA	57.58 $\pm$ 1.76 fgh
EB	63.64 $\pm$ 2.39 cdef
EC	67.68 $\pm$ 1.70 bcdef
ED	52.53 $\pm$ 2.96 ghi
EE	40.40 $\pm$ 1.02 j
EF	67.68 $\pm$ 4.99 bcdef
EG	61.62 $\pm$ 0.83 defgh
EH	62.63 $\pm$ 2.70 defg
EK	64.65 $\pm$ 0.66 bcdef
EL	65.66 $\pm$ 4.01 bcdef
EM	71.72 $\pm$ 1.64 abcd
EN	70.71 $\pm$ 4.81 bcde
EO	46.46 $\pm$ 1.72 hi
EP	51.52 $\pm$ 1.57 ij
EQ	59.60 $\pm$ 4.73 fgh
ER	63.64 $\pm$ 2.39 cdef
EZ	63.64 $\pm$ 5.26 cdef
FS1	62.63 $\pm$ 2.86 defg
FS2	61.62 $\pm$ 3.83 defgh
FS3	51.52 $\pm$ 6.48 hi
FS4	57.58 $\pm$ 8.24 fgh
P1	39.39 $\pm$ 0.65 j
P4	51.52 $\pm$ 1.57 hi
P5	51.52 $\pm$ 6.66 hi
P7	46.46 $\pm$ 2.71 ij
PC1	61.62 $\pm$ 3.71 defgh
PC2	62.63 $\pm$ 9.93 defg
SP0	65.66 $\pm$ 2.54 bcdef
SP1	71.72 $\pm$ 0.38 abcd
SP2	67.68 $\pm$ 2.15 bcdef
SP3*	80.81 $\pm$ 1.23 a
SP4	64.65 $\pm$ 5.16 bcdef
SP5	66.67 $\pm$ 2.16 bcdef
TH	67.68 $\pm$ 0.05 bcdef

Note: According to Duncan's test, the average value followed by the same letters in each row is not significantly different at  $P < 0.05$ . \*indicating strain with the highest inhibition value.





**Figure 1.** Observation of *Pyricularia oryzae*: (A) symptom caused by *Pyricularia oryzae* on rice leaf (indicated by the arrow); (B) *Pyricularia oryzae* at 7 days on OMA; (C) conidiophore of *Pyricularia oryzae*



**Figure 2.** *In vitro* antagonistic assay between endophytes and fungal pathogens: (A) SP3 antagonistic to *Pyricularia oryzae*; (B) D11 antagonistic to *Pyricularia oryzae*; (C) D8 antagonistic to *Pyricularia oryzae*

**Table 2.** Molecular identification of fungi isolates based on the alignment of ITS region sequences

Fungal isolates		Nearest phylogenetic neighbor	Similarity (%)	Taxonomical assignment	Accession number
Pathogen	PY	<i>Magnaporthe oryzae</i> isolate PMB2	99.76	<i>Pyricularia oryzae</i>	MG407029.1
Endophytes	SP3	<i>Trichoderma asperellum</i> isolate Tasum66	100	<i>Trichoderma asperellum</i>	MT102403.1
	D11	<i>Curvularia chiangmaiensis</i> strain LC12044	100	<i>Curvularia chiangmaiensis</i>	MN215651.1
	D8	<i>Fusarium solani</i> isolate HJ12	99.81	<i>Fusarium solani</i>	MG827183.1

Morphological observation of endophytic fungi (Figure 3) after five days in culture showed that *T. asperellum* had a green mycelium with a rough texture on the colony's top, while the bottom of the mycelium was white (Figure 3.A). This fungus had a relatively high growth ability and can grow to cover the plate (diameter 9 cm) within 4 days after inoculation on PDA media. Morphological observation of *T. asperellum* showed that conidia were globose and clustered, with sizes from 2.5  $\mu\text{m}$  to 2.8  $\mu\text{m}$  (Figure 3.B). It was also seen that fungus had branched conidiophores (Figure 3.C). Observation of the *C. chiangmaiensis* fungus showed a black mycelium base color and a grayish surface color, with umbonate elevation and irregular edges (Figure 3.D). The conidia were curved with three septate parts, and one central cell was larger than the other cells. The conidia were dark brown in color, averaging 18-23  $\mu\text{m}$  in length, 7.8-9.2  $\mu\text{m}$  in width, and the septate hyphae (Figure 3.E-F). *Fusarium solani* had a white mycelium color with a fine texture (Figure 3.G). The macroconidia had mean lengths of 31-33  $\mu\text{m}$   $\times$  3.9-4.2  $\mu\text{m}$ , and are curved with 3-4 septate parts (Figure 3.H-I).

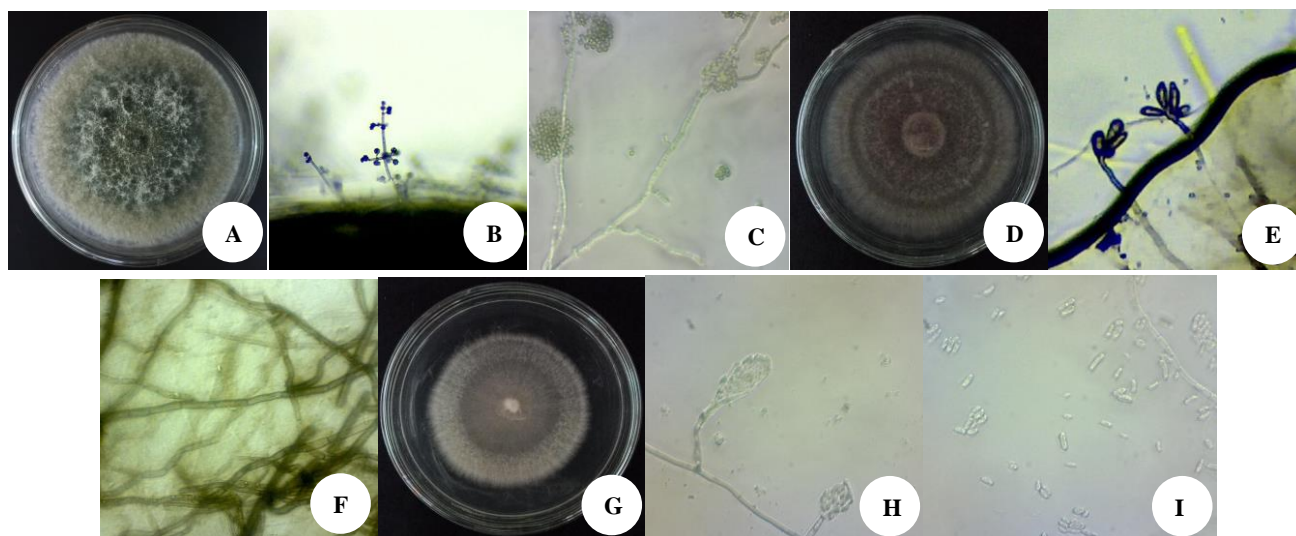
#### Scanning electron microscope

Result of SEM revealed that the interaction between the pathogens and endophytes showed complexity under

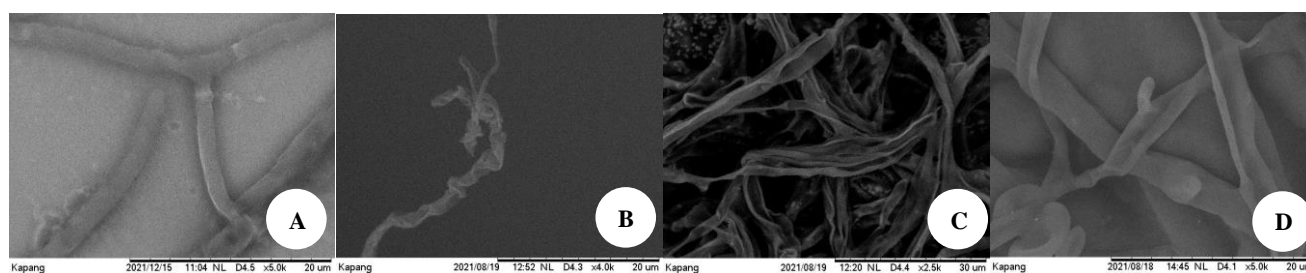
investigation. It was found that the hyphae of *P. oryzae* became turgid and collapsed (Figure 4.B, C, D) compared to control (Figure 4.A). In addition, the growth of the tips of the hyphae became abnormal and stunted.

#### Quantification of hydrolytic enzymes production

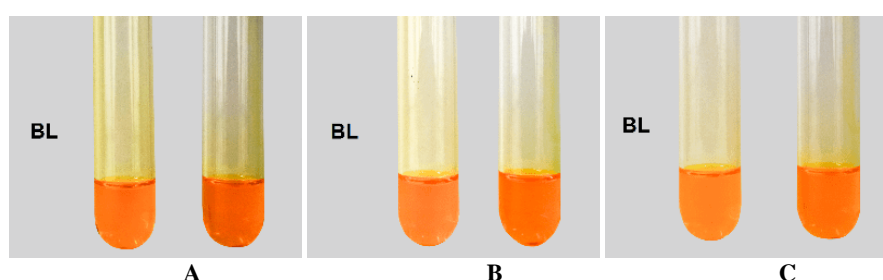
A colorimetric method was employed to study the activity and the amount of enzymes produced by the endophytic fungi. In a certain concentration range, the amount of reducing sugar and the color from orange to brown present a positive correlation (Figures 5 and 6). The results of chitinase and cellulase concentrations by endophytic fungal isolates were obtained from the calculation of the regression equations obtained from the standard curve. Blanks (control) were used to set the spectrophotometer at zero absorbance. The obtained regression equations were  $y = 0.2977x - 0.0871$  for the chitinase assay and  $y = 0.0393x - 0.0102$  for the cellulase assay. The concentration of chitinase and cellulase from endophytic fungi isolates are shown in Table 3. The results showed a significant difference in cellulase quantification ( $F = 5.19$ ,  $P = 0.04$ ) and insignificant difference in cellulose quantification ( $F = 0.84$ ,  $P = 0.48$ ).



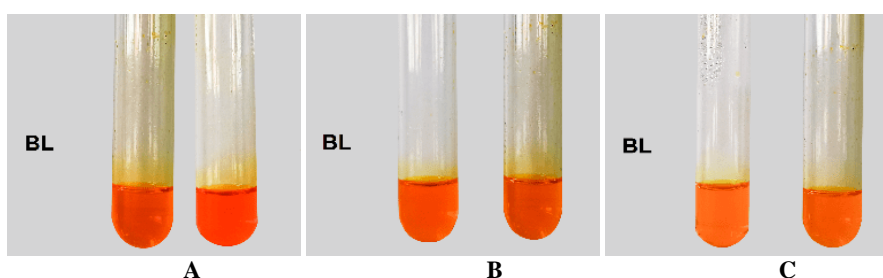
**Figure 3.** Morphology characteristics of endophytic fungi: A. Growth of *Trichoderma asperellum* on PDA media; B. Conidiophore of *Trichoderma asperellum*; C. Conidia of *Trichoderma asperellum*; D. Growth of *Curvularia chiangmaiensis* on PDA media; E. Conidiophore of *Curvularia chiangmaiensis*; F. Hyphae of *Curvularia chiangmaiensis*; G. growth of *Fusarium solani* on PDA media; H. Conidiophore of *Fusarium solani*; I. Macroconidia of *Fusarium solani*



**Figure 4.** Scanning electron microscope analysis of *Pyricularia oryzae* hyphae: A. Control B. Hyphae of *Pyricularia oryzae* inhibited by *Trichoderma asperellum*, B. Hyphae of *Pyricularia oryzae* inhibited by *Curvularia chiangmaiensis*, C. Hyphae of *Pyricularia oryzae* inhibited by *Fusarium solani*



**Figure 5.** Endophytic fungus supernatants after adding DNS reagent in chitinase quantitative assay: BL: Blank, A: *Trichoderma asperellum*, B: *Curvularia chiangmaiensis*, C: *Fusarium solani*



**Figure 6.** Endophytic fungus supernatants after adding DNS reagent in cellulase quantitative assay: BL: Blank, A: *Trichoderma asperellum*, B: *Curvularia chiangmaiensis*, C: *Fusarium solani*

**Tabel 3.** Enzymatic concentration produced by endophytic fungi

Endophytic fungi	Chitinase concentration ( $\mu\text{g/mL NAG}$ )	Chitinase activity (U/minute)	Cellulase concentration ( $\mu\text{g/mL glucose}$ )	Cellulase activity (U/mL)
<i>Trichoderma asperellum</i>	1.51 $\pm$ 0.07 a	0.25 $\pm$ 0.01 a	4.09 $\pm$ 0.50 a	0.68 $\pm$ 0.08 a
<i>Curvularia chiangmaiensis</i>	1.43 $\pm$ 0.12 a	0.24 $\pm$ 0.02 a	1.83 $\pm$ 0.68 b	0.30 $\pm$ 0.11 b
<i>Fusarium solani</i>	1.46 $\pm$ 0.04 a	0.24 $\pm$ 0.01 a	3.84 $\pm$ 1.40 a	0.64 $\pm$ 0.23 a

Note: According to Duncan's test, the average values followed by the same letters within each row are not significantly different at  $P < 0.05$ .

## Discussion

The observation of *P. oryzae* symptoms in this study are in accordance with previous studies by Ghatak et al. (2013) who reported that the preliminary symptoms of rice blast are oval lesions that are 0.30-0.50 cm wide and 1.00-1.50 cm long, colored from white to gray color, and surrounded by a dark border. Old lesions are usually large and can grow together to damage the entire leaf. Kulkarni and Peshwe (2019) studied the growth and sporulation of *P. oryzae* on different media formulations. They found that *P. oryzae* in OMA medium had cottony mycelia with black pigmentation, peripheral cottony white mycelia, and irregular margins. Kariaga et al. (2016) reported that the conidia of *P. oryzae* are typically pyriform with a rounded bottom, 2 until 3 septate parts, narrowed apex, 2 until 4 cells, and center cells broader than others. The length of the conidia varied from 22.30 to 38  $\mu\text{m}$ . The aforementioned characteristics were in accordance with those observed in this study. In the fungal antagonist assay, selected endophytic fungi showed inhibitory mechanisms, such as mycoparasitism and antibiosis. Kohl et al. (2019) described parasitism as an immediate aggressive interaction between two organisms wherein one organism gets nutrient from the other. If the host is likewise a parasite, or phytopathogen, this interaction is described as hyperparasite. This is supported by the description of Ruiz et al. (2020) that mycoparasitism is the direct invasion of a fungus (target) through another fungus (the mycoparasite) that aims to utilize the target as a food source. The important phases of the process are prey detection and chemical and physical aggression. The association of the two is typically for fungal prey hyphal killing and ultimately nutrient uptake. Pre-contact chemical attacks involve the triggered production and mass launch of cell wall-degrading enzymes. The antibiosis mechanism produces a halo zone between the antagonist and pathogenic fungus. Awad et al. (2012) explained that a halo zone (clear zone) can be formed due to the presence of antimicrobial activity produced by antagonist microbes.

Molecular identification of fungi was performed using the universal primers ITS 1 and ITS 4. The nuclear ribosomal RNA internal transcribed spacer (ITS) region has been shown to be the most likely to be correctly identified for the numerous fungal strains that were analyzed and the most clearly defined barcode gaps (Badotti et al. 2017). The ITS region is a quite polymorphic non-code area with sufficient classification units. Accordingly, the sequences can be separated at the species level. It is positioned on the inside of the operon of

ribosomal RNA, and the length ranges from 450 to 750 bp (Beeck et al. 2014). Based on each sequence, endophytic fungi were identified as *T. asperellum*, *C. chiangmaiensis*, and *F. solani*. The highest inhibition ability strain was isolated from rice plant endophytes. The result of morphological characteristics of endophytic fungi is in accordance with a previous studies by Wu et al. (2017), in which it was noted that the aerial part of the *Trichoderma* colony had a dark green spore color (on the top of the colony) and white mycelium color (on the reverse part). Mycelial morphology is rough, and dark, inexperienced spores start to form at high rates within and inside the colony's center. Mishra et al. (2016) reported that *T. asperellum* species grown on PDA media had a dark yellowish green colony color, smooth colony edge, cottony white mycelial color, and smooth mycelial form. Prakasapandian et al. (2016) revealed that *T. asperellum* had regularly branched, typically paired conidiophores with phialides. The conidia were globose to sub-globose in shape and had a measurement from 2.56  $\mu\text{m}$  to 3.09  $\mu\text{m}$  in length and from 2.15  $\mu\text{m}$  to 2.62  $\mu\text{m}$  in width. Marin et al. (2018) mentioned that *C. chiangmaiensis* hyphae were hyaline to light brown, branched, and septate. The conidia were curved and expanded unevenly from the central cell; they also ellipsoidal to obovoid and light brown to brown, with apical and basal cells barely thinner than the central cell, 2-3 distoseptate parts, and sizes of 17-23.50  $\times$  9-12.50  $\mu\text{m}$ . Despite their characteristics in culture, *C. chiangmaiensis* colonies on PDA media reached 65-70 mm diameter in a week, with sparse hyphae and slightly leafy edges as well as a chestnut surface with umber edges. Tan et al. (2021) explained that *Fusarium* species have three types of spores: macroconidia, microconidia, and chlamydospores. *Fusarium solani* isolates have aerial mycelium pigmentation from white to cream, while the colony varied from white to brown. Microscopic observation showed that the mycelium was branched, hyaline, and septate. The macroconidia had 3-4 septate hyaline parts and sizes of 21-28  $\times$  3.8-4.5  $\mu\text{m}$ . Hami et al. (2021) observed that *F. solani* mycelium was smooth, cylindrical, branched, and septate. The conidiophores measured 88.60-110.50  $\times$  2.5-4.50  $\mu\text{m}$  in size and were cylindrical and septate. *Fusarium solani* has two types of conidia: microconidia and macroconidia. The microconidia were hyaline and septate, had round to oval shape, and measured 7.50-11.00  $\times$  2.80-3.75  $\mu\text{m}$ . The macroconidia were curved and hyaline, had 3-4 septate parts, and measured of 21.15-32.00  $\times$  3.80-4.75  $\mu\text{m}$ .

The study of endophytic fungi revealed major changes in cell wall of the pathogen. SEM observations showed that pathogen cell wall was disintegrated and experienced loss of cell turgor, although the hyphae of endophytic fungi did not interact physically with the pathogen hyphae by coiling around or penetrating. However, the wrinkling and collapse of *P. oryzae* hyphae can be caused by wall-lytic enzymes or antifungal substances secreted by endophytic fungi. Enzymatic activity of *T. asperellum*, *C. chiangmaiensis*, and *F. solani* has been detected in some experiments. This activity may induce cell wall damage and degradation. Sha et al. (2020) reported that *P. oryzae* P131 cells treated with *Bacillus pumilus* strain S9 and *Bacillus amyloliquefaciens* strain S170 had peculiar cell morphology and disruption, indicating that they degrade the cell walls, destroy cell membranes, and damage cellular organelles. In the measurement of chitinase, the standard curve created by NAG was used to determine the reducing sugar concentration of NAG released in colloidal chitin-supplemented medium (Agrawal and Kotasthane 2012). The suppression of pathogen growth by *T. asperellum* is directly correlated with the mycoparasitic properties from antagonistic fungi. *Trichoderma asperellum* showed the highest concentration of chitinase production and the highest suppression ability. The most well-studied mycoparasites belong to the genera *Trichoderma* and *Clonostachys*. Individual strains of antagonistic fungal isolates from these genera usually have many phytopathogenic hosts and different host ranges. The structures for attachment and infection are produced by antagonist isolates, frequently in combination together with antimicrobial secondary metabolites, killing the host with cell wall-degrading enzymes (Karlsson et al. 2017; Nygren et al. 2018). On the other hand, *F. solani* produces almost the same amount of chitinase as *T. asperellum*. Laien and Mohammadi (2021) demonstrated that the presence of chitinase genes and/or the ability to produce chitinolytic enzymes had been proven in 83 *Fusarium* isolates detected by the chitinase bioassay method and/or DECH primers for chitinase. Replicated regions of some *Fusarium* isolates had high similarities to sequences of chitinase genes in *Trichoderma* species. This study demonstrated the mycoparasitism of the endophytic fungal complex against *P. oryzae* through enzymatic production. Chitinase plays an important role in the biocontrol of many fungal plant pathogens by lysing fungal cell walls. Chitinase degrades the chitin polymers present in the cell walls of plant phytopathogenic fungi (Veliz et al. 2017; Iqbal and Anwar 2019). In addition, in other fungal systems, chitinases play a key role in development and branching of fungal hyphae, spore production and germination, cell division and degradation of the fungal cell wall, and morphogenesis of the cell wall structure (Hartl et al. 2012). For this reason, introducing of the fungal chitinase gene to plants may result in increased resistance to phytopathogenic chitinous fungi (Mythili et al. 2018). All endophytes have the ability to produce cellulose, one of the important enzymes responsible for cell wall degradation. Cellulase plays a key role in nature by catalyzing the hydrolysis of the 1,4- $\beta$ -D-glycosidic bond in cellulose and recycling this

polysaccharide (Jadhav et al. 2017). Cellulose hydrolysis may occur through physical, chemical, and enzymatic processes. Enzymes produced by fungi are prominent in most of the enzymatic processes (Onofre et al. 2015). Cellulase enzymes form an enzymes complexe that contains three primary types of synergistically active enzymes: exo- $\beta$ -1,4-glucanases, endo- $\beta$ -1,4-glucanases, and  $\beta$ -glucosidase (Strakowska et al. 2014). As with different organisms that invade plant tissues, endophytes produce extracellular hydrolyzing enzymes as a mechanism to resist pathogen invasion and to obtain nutrition from the host (Sunitha et al. 2013). Microorganisms play an significant function in the degradation of cellulose and hemicellulose, they stand out from endophytes, which are an excellent source of hydrolases. During the endophyte period, the usage of these enzymes must clearly be associated with mutualistic courting with the host plant (Robl et al. 2013).

In this study, three of the 50 rice endophytic fungi isolates have the highest ability to inhibit *P. oryzae*. They were identified according to ITS sequences as *T. asperellum*, *C. chiangmaiensis*, and *F. solani*. They are able to produce hydrolytic enzymes, such as chitinase and cellulase, which function to degrade the cell walls of pathogenic fungi. *Pyricularia oryzae* hyphae were seen to be abnormal in the inhibitory assay by endophytic fungi. However, further research needs to be done on other inhibitory compounds by these endophytic fungi. This study can provide information on endophytic fungal species that can be used as biocontrol agents in control blast disease in rice caused by *P. oryzae*.

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