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Research Article
Exploring Fungal Candidates from Aromatic Rhizosphere as Biological Controls Against Three *Pyricularia oryzae* Haplotypes

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Abstract

Background and Objective: Blast disease (*Pyricularia oryzae*) is a major disease-causing yield losses in rice crops worldwide. Disease control using resistant varieties is less effective due to the high genetic variation in *P. oryzae* populations in the field and the use of synthetic fungicides hurts the diversity of biological agents. This study aims to explore fungi in the rhizosphere of organic aromatic rice in North Luwu Regency that can utilized as biological control agents against three haplotypes of *P. oryzae*. **Materials and Methods:** Isolation of rhizosphere fungi using serial dilution method and scatter plate method. The identification of fungi based on microscopic and macroscopic characteristics. Genotype test of 15 *P. oryzae* isolates used gene-based markers related to virulence traits, namely Erg2 (1,440 bp), Pwl2 (900 bp) and Cut1 (1,730 bp). Amplified DNA bands that appeared were scored as 1 (present) and 0 (absent). **Results:** Exploring organic rice rhizosphere fungi in North Luwu Regency found potential biological control agents against three *P. oryzae* haplotypes on local varieties: Juvenile and Bandarata. Twelve fungal isolates from the rhizosphere of aromatic rice were successfully isolated and six antagonistic fungal isolates were able to inhibit the growth of *P. oryzae* haplotypes C-011, D-111 and F-110. *Trichoderma* spp., isolates had the highest inhibition percentage of 72-90%, followed by *Penicillium* sp., 1 with an inhibition percentage of 62-82%. **Conclusion:** Twelve fungal isolates from the rhizosphere of aromatic rice were successfully isolated and six antagonistic fungal isolates were able to inhibit the growth of *P. oryzae* haplotypes C-011, D-111 and F-110.

Key words: Antagonistic fungi, aromatic rice, haplotypes, plant disease control, rhizosphere fungi

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pyricularia oryzae Cavara, also known as *Magnaporthe oryzae*, causes blast disease, a significant threat to rice crops[1,2], infecting various aboveground tissues throughout all growth stages and potentially leading to total crop loss. Lesions produced by this pathogen on leaves, stems and panicles vary in appearance based on factors such as varietal resistance, environmental conditions and plant age[3].

Blast disease retrieved to cause 100% yield loss in Japan and Brazil, while in India, Korea and China it ranges between 4 and 14% and in the Philippines 50 and 85%[4]. It has been identified as attacking four rice varieties in Kutai[5]. Blast disease outbreaks in Indonesia can cause yield losses of around 50-90% in susceptible rice plants[6]. Host and environment cause natural genetic changes so that new races emerge[7] able to change the virulence properties of the pathogen in a short time[8]. Race changes are influenced by differences in rice cultivars grown in a region[9]. Dominant races in the same of region can change over time depending on the growing season and changing ecosystems[10] or changes in genetic composition and high mutation rates in the blast fungus[11]. Race 033, 133 and 173 are the important races of *P. oryzae* that frequently infect rice fields in Indonesia[12] and race 001 is the most dominant in Indonesia[11]. Five different haplotypes found, namely haplotypes A-000 (4 isolates), C-011 (3 isolates), D-111 (2 isolates), F-110 (3 isolates) and G-100 (3 isolates) in seven differential rice varieties, Asahan, Cisokan, IR 64, Krueng Aceh, Cisadane, Cisanggarung and Kencana Bali[13], high genetic variation of blast pathogens[14], making it difficult for pathogens to be controlled.

In general, farmers use synthetic fungicides to suppress blast disease attacks but they are not effective. Less than 0.1% of pesticides applied for pest control effectively reach their intended target, with over 99.9% migrating into the environment, posing risks to public health, biota and ecosystem integrity by contaminating soil, water and air. Hence, exploiting microbes from organic rice plant rhizospheres as biological agents holds significant potential[15].

One of the environmentally friendly controls is biological control based on antagonistic microorganisms. Antagonistic microorganisms as biological agents have a high potential in inhibiting pathogen attacks directly or indirectly[16].

The use of local rice varieties, high adaptability and specific characteristics including microorganisms in the rhizosphere is a potential source of biological agent development. Local aromatic rice varieties are organically cultivated and have a distinctive aroma is cultivated in the Rongkong District. It is a remote area located at an altitude of 1,000-1,800 m above sea level and it is located in the border triangle between the provinces of South Sulawesi, West Sulawesi and Central Sulawesi. Local aromatic rice in North Luwu Regency consists of Bandarata, Banjara, Dambo, Jamborana, Paresale, Parekamba, Remaja and Tarone varieties with a plant age of up to six months. Organic cultivation of local rice is expected to maintain the abundance of biological agents in the rhizosphere, especially biocontrol, bioindicators and biofertilizers. The utilization of biological agents to suppress the growth of pathogenic fungi has been widely practiced, as it has a positive impact on the environment. The application of biological agents leaves no residue and does not cause plant resistance to disease[17]. Meanwhile, for plants, biological agents can suppress the growth of pathogens. Biotic and abiotic factors play a role in the survival of biological control agents such as temperature, acidity, humidity and several other components[5] biological agent treatment was able to reduce the percentage of disease severity and reduced the and reduce the percentage of leaves attacked by blast disease[18].

Rhizosphere fungi have an important role as biological control agents because they can suppress primary infection of a pathogen through their ability to produce antibiotics[16], chitinase enzymes and cellulase enzymes, thus isolating and identifying microbes from organic aromatic rice plants that live in natural ecosystems and it is expected to obtain diverse species of antagonistic fungi. Therefore, it was important to collect antagonistic isolates from the rhizosphere of local aromatic rice and test their ability as biological control agents on three haplotypes of blast disease (*P. oryzae*) *in vitro*.

MATERIALS AND METHODS

Study area

Location of aromatic rice rhizosphere fungi: The locations of rhizosphere samples taken from Salurante, Rongkong District, North Luwu Regency. Sampling was conducted from September to November, 2023.

Isolation of rice rhizosphere fungi: Fungal isolation was carried out using the serial dilution method. While 10 g of soil was weighed and diluted with 90 mL of distilled water. Serial dilutions using 1 mL of the final concentration of 10^4 were poured on solid PDA media and incubated at 30°C for 3-7 days. The growing fungal colonies were transferred to new PDA media[19].

Identification of rice rhizosphere fungi: Identification of rhizosphere fungi was carried out by observing macroscopic and microscopic morphological characters. Macroscopic observations of fungi include the color and surface of colonies, radial lines from the center of the colony towards, the edge of the colony and concentric circles. The identification of fungi refers to the book Illustrated Genera of Imperfect Fungi[20]. Microscopic morphological observations under a 40x magnification microscope (Olympus CX 23 LED). The microscopic characteristics observed were reproductive structures (presence or absence of rhizoids, conidia and spores) and hyphal structures.

Virulence gene detection of *Pyricularia oryzae*: Isolation of genomic DNA of *P. oryzae* fungal isolates from fungal mycelial cultures[21]. The DNA extraction by following the procedure of gSYNC™ DNA Extraction Kit as much as 25 mg. The fungal mycelia were transferred into a 1.5 mL centrifugation tube then the mycelia were centrifuged for 5 min, 200 µL GST Buffer and 20 µL protease K were added then the mycelia were crushed using a micro pestle then vortexed quickly. Next, incubate overnight at 60°C until the sample becomes lysate. If there are still clumps after incubation, then centrifugation is carried out for 2 min at 14-16,000×g, the supernatant is transferred to a new 1.5 mL microcentrifugation tube and Buffer then vortexed 200 µL of absolute ethanol is added to the sample and vortexed moderately for ±10 sec the sample is then poured into a GD column in a 2 mL collection tube and centrifuged. The DNA collected in the GD column was then added to 400 µL w1 Buffer and then centrifuged for 30 sec until pure DNA was obtained. Amplification was carried out using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystem USA) and cycles ran following[22]. Genotype testing of *P. oryzae* fungus was carried out using gene-based markers for virulence traits, namely Cut1, Erg2 and Pwl2 markers, which are virulence-related. Identification was carried out by PCR with a pair of specific primers for the Erg2 (1,440 bp), Pwl2 (900 bp) and Cut1 (1,730 bp) genes. The PCR reaction used Go taq® Green Master mix enzyme which contained 2x green Go Taq® reaction buffer (pH 8.5) 400 µL dATP, dGTP, 400 µM dTTP and 3 µM $MgCl_2$, H_2O and 3 µL DNA template[22]. The base sequence of the primers is Cut1 (F: 5'-TATAGCGTTGACCTTGTGGA3'; R: 5'TAAGCATCTCAGACCG AACC-3'), Erg2 (F: 5'-GCAGGGCTCATTCTTTTCT3'; R: 5'CCGAC TGGAAGGTTTCTTTA-3') and Pwl2 (F: 5'-TCCGCCACTTTT CTCATTCC-3'; R: 5'-GCCCTCTTCTCGCTGTTCAC-3'). The PCR programmed started with initial denaturation at 94°C for 30 sec, 54°C for 30 sec, 72°C for 1 min and the last cycle at 72°C for 5 min. Electrophoresis of PCR products was carried out on a 1% (w/v) agarose gel to which 6 µL of florosafe DNA stain was added as an alternative DNA dye to Ethidium Bromide (EtBr) with an electrical voltage of 100 Volts for 40 min to identify amplicons for each primer according to the reference size[22,23]. Amplified DNA bands that appear for each primer from each isolate are scored with a value of 1 (present) and 0 (absent). Furthermore, this data was used to group the test isolates into several haplotypes, based on the combination of the three types of genes (Pwl2, Erg2 and Cut1) in the tested isolates.

Antagonistic test of rhizosphere fungi against pathogenic fungi: Selection of rice rhizosphere fungi through antagonistic tests against *P. oryzae* using the dual culture method on each isolate with three replicates. The control treatment was *P. oryzae* inoculated into PDA media without treatment. Diagonal lines were drawn on the outer surface of the Petri dish[24]. The spacing is 3 cm between fungi and 3 cm to the edge of the Petri dish. Measurement of the growth radius of *P. oryzae* started from 1-12 days after inoculation. Observations were stopped when the control colonies reached maximum growth with an interval of 2 days to observe the percentage of growth inhibition of *P. oryzae* by antagonistic fungi. The following is the calculation formula for percentage inhibition of radial growth (PIRG)[25]:

$$P\ (\%) = \frac{R_1 - R_2}{R_1} \times 100$$

Where:

- P = Inhibition percentage (%)
- R_1 = Radius of pathogenic fungi without rhizosphere fungi (control)
- R_2 = Radius of pathogenic fungi with rhizosphere fungi

Determination of the level of inhibition based on percentage inhibition of radial growth (PIRG) and Bell rating[26,27] classified PIRG as follows: (i) PIRG<50%: Low, (ii) 50%<PIRG<60%: Medium, (iii) 60%<PIRG<75%: High and (iv) PIRG>75%: Very high (Table 1). From the results of *in vitro* antagonistic tests, isolates of rice rhizosphere antagonistic fungi that have the potential to inhibit the growth of *P. oryzae* will be obtained.

Table 1: Bell rating scheme[26]

Rating	Description
1	Antagonist completely goes beyond the limits of the pathogen and covers the entire surface of the substrate
2	Antagonists control 67% of the media surface
3	Each antagonist and pathogen occupy 50% of the surface of the medium and no other organism dominates
4	Pathogen manages to surpass the antagonist and control 67% of the surface of the medium
5	Pathogen completely masters the surface of the substrate

Data analysis: Amplified *P. oryzae* DNA bands that appeared on each primer (Pwl2, Erg2 and Cut1) were scored with a value of 1 (present) and 0 (absent), then grouped into several haplotypes. Data from the antagonist test and rhizosphere fungi were statistically analyzed using Analysis of Variance (ANOVA) to determine whether there was an effect of rice rhizosphere fungi on pathogens in the antagonist test if there was an effect, then Duncan's further test was carried out, at a significant level ($\alpha = 0.05$).

RESULTS

Rhizosphere fungi of local aromatic rice of North Luwu Regency: Fungal isolates AR1, AR3-AR9 were obtained in rhizosphere samples of rice of the Remaja and Bandarata variety while fungal isolates AR2, AR10, AR11 and AR12 were only found in rhizosphere samples of rice of the Bandarata variety (Fig. 1).

***Pyricularia oryzae* haplotype analysis:** The 15 isolates were successfully amplified using primer Pwl2 8 isolates, 9 isolates were successfully amplified using primer Erg2 and 5 isolates were successfully amplified using primer Cut1. Variations of virulence gene DNA bands amplified from each isolate were obtained (Fig. 2). Electrophoresis results which represent each haplotype obtained as many as 5 haplotypes including haplotype A-000 (4 isolates), haplotype C-011 (3 isolates), haplotype D-111 (2 isolates), haplotype F-110 (4 isolates) and haplotype G-100 (2 isolates) (Table 2).

Macroscopic and microscopic of three *Pyricularia oryzae* haplotypes (C-011, D-111, F-110): *Pyricularia oryzae* has a colony surface growth pattern forming concentric rings that lead to the center, grey to blackish colonies during the incubation period of 6-12 days, velvety colony texture, pyriform conidia shape which is rounded at the base and narrowed at the end, colorless (hyaline) and there are two septa or three chambers (Fig. 3-5).

***In vitro* testing of rice rhizosphere fungi against three *Pyricularia oryzae* haplotypes:** Based on the analysis of variance, at the observation of 4-12 days after inoculation (dai) there was a significant difference between the control and the rhizosphere fungus treatment. Observations for 12 days were stopped because the control had filled the Petri dish and the rice rhizosphere fungi were no longer actively inhibiting. Six isolates of rhizosphere fungi had $\geq$50% inhibition against *P. oryzae* haplotype C-011, D-111 and F-110 (Table 3-5), namely AR1-AR5 and AR7. Inhibition of rhizosphere fungi against the growth of *P. oryzae* haplotype C-011 (Table 3), treatment AR1 showed the highest inhibition was 90%, followed by treatment AR3 (82%), AR4 (73%), AR5 (71%), AR7 (63%) and AR2 (60%). The ability of rhizosphere fungal isolates to inhibit the growth of *P. oryzae* D-111 (Table 4), treatment AR1 has the highest inhibition of 72%, followed by AR4 (65%), AR5 (64%), AR3 (62%), AR7 (55%) and AR2 (51%). Inhibition of rhizosphere fungal isolates against the growth of *P. oryzae* haplotype F-110 (Table 5), AR 1 treatment showed the highest inhibition of 83%, then AR3 (73%), AR4 (63%), AR2 and AR5 each 55% and AR7 inhibition was 53%.

Identification of antagonistic fungi from the rice rhizosphere: Identification of rhizosphere fungal isolates was carried out on isolates that had the highest percentage of inhibition in suppressing the growth of *P. oryzae* haplotypes C-011, D-111 and F-110. The identification results in Fig. 1 and Table 6 show six isolates of antagonistic fungi in the genus *Trichoderma* sp., *Paecilomyces* sp., *Penicillium* sp. and *Aspergillus* sp. Isolate AR1 is *Trichoderma* sp., has a green and white colony color, rough texture, uneven edges, a round conidia shape and a branched conidiophore or conidia stalk. Isolate AR2 is *Paecilomyces* sp., has pink colonies at the beginning of growth and as the incubation period increases the color is slightly brownish purple conidia are formed in a series of chains and the base of the phialide is larger and gradually tapers towards the apex and is rather long and slender, also forming a brush (*Penicillus*). Isolates AR3 and AR7 were *Penicillium* sp., characterized by the shape of round conidia at the end of a series of conidiophores, upright and branched conidiophores resembling branching bushes and at the ends there is a collection of cylindrical phialids, greenish colonies with a red base and greenish colonies with a yellow base. Isolate AR4 is *Aspergillus niger* characterized by brown to black colony color and has round conidia and vesicles and long and cylindrical conidiophores, while isolate AR5 is *Aspergillus flavus* characterized by green colony color and round conidia, round vesicles and long and cylindrical conidiophores.

Table 2: Grouping of *P. oryzae* haplotypes based on the presence of virulence genes

	Presence amplicons			
Isolate code	Pwl2[a]	Erg2[b]	Cut1[c]	Haplotype
1	1	1	0	F-110
2	0	1	1	C-011
3	0	0	0	A-000
4	1	1	1	D-111
5	0	1	1	C-011
6	0	0	0	A-000
7	1	1	1	D-111
8	1	1	0	F-110
9	1	1	0	F-110
10	0	0	0	A-000
11	1	1	0	F-110
12	0	0	0	A-000
13	1	0	0	G-100
14	1	0	0	G-100
15	0	1	1	C-011

[a]800-900 bp, [b]1.440 bp and [c]800-1.730 bp

Table 3: Inhibition of rhizosphere fungi against *P. oryzae* haplotype C-011

	Inhibition (%) dai			
Isolate code	4	8	12	Bell rating
AR1	43^{g}	75^{f}	90^{f}	1
AR2	30^{bcd}	53^{cd}	60^{c}	2
AR3	41^{g}	62^{e}	82^{e}	1
AR4	37^{f}	57^{de}	73^{d}	2
AR5	34^{def}	48^{c}	71^{d}	2
AR6	27^{b}	35^{b}	45^{b}	4
AR7	34^{ef}	53^{cd}	63^{c}	2
AR8	36^{f}	39^{b}	41^{b}	4
AR9	32^{cde}	40^{b}	45^{b}	4
AR10	29^{bc}	37^{b}	44^{b}	4
AR11	30^{bcd}	37^{b}	43^{b}	4
AR12	30^{bcd}	38^{b}	44^{b}	4
Control	0^{a}	0^{a}	0^{a}	5

Numbers followed by the same letter in the same column do not show significant differences in the Duncan's test at the 5% level

Table 4: Inhibition of rhizosphere fungi against *P. oryzae* haplotype D-111

	Inhibition (%) dai			
Isolate code	4	8	12	Bell rating
AR1	39^{e}	53^{g}	72^{f}	2
AR2	18^{c}	36^{de}	51^{d}	3
AR3	29^{d}	50^{g}	62^{e}	2
AR4	41^{e}	42^{f}	65^{e}	2
AR5	30^{d}	40^{ef}	64^{e}	2
AR6	23^{c}	26^{b}	42^{bc}	4
AR7	21^{c}	39^{ef}	55^{d}	3
AR8	37^{e}	38^{ef}	38^{b}	4
AR9	7^{b}	32^{cd}	40^{bc}	4
AR10	19^{c}	31^{bc}	39^{bc}	4
AR11	23^{c}	31^{cd}	43^{c}	4
AR12	21^{c}	30^{bc}	40^{bc}	4
Control	0^{a}	0^{a}	0^{a}	5

Numbers followed by the same letter in the same column do not show significant differences in the Duncan's test at the 5% level

Table 5: Inhibition of rhizosphere fungi against *P. oryzae* haplotype F-110

	Inhibition (%) dai			
Isolate code	4	8	12	Bell rating
AR1	31[d]	61[h]	83[f]	1
AR2	22[ab]	37[de]	55[c]	3
AR3	27[cd]	53[g]	73[e]	2
AR4	41[e]	60[h]	63[d]	2
AR5	23[ab]	45[f]	55[c]	3
AR6	30[d]	23[b]	38[b]	4
AR7	23[ab]	37[d]	53[c]	3
AR8	31[d]	40[e]	39[b]	4
AR9	17[b]	31[c]	38[b]	4
AR10	29[cd]	31[c]	37[b]	4
AR11	30[d]	31[c]	37[b]	4
AR12	31[d]	29[c]	37[b]	4
Control	0[a]	0[a]	0[a]	5

Numbers followed by the same letter in the same column do not show significant differences in the Duncan's test at the 5% level

Table 6: Morphological characteristics of rhizosphere fungal isolates of rice remaja and bandarata varieties

	Macroscopic		Microscopic		
Isolate code	Surface above	Surface below	Hifa	Conidia/Spora	Genus
AR1	Dark green and white, rough texture and uneven edges	Greenish-white	Septa, hyaline, branched	Short conidiophores, branched and rounded conidia	*Trichoderma* sp.
AR2	Pink with white edging, radial colonies, flat edges and smooth texture	Yellowish white	Septa, hyaline, branched	Spherical conidia, branched conidiophores like shrubs and apex phialids tapered	*Paecilomyces* sp.
AR3	Blackish-green, radial colony, flat edges and starchy texture	Red	Septa, hyaline, branched	Spherical conidia, sepat conidiophores and branched like shrubs	*Penicillium* sp. 1
AR4	Black and white, granular and compact colony shape, flat edges and smooth texture	White	Non-septa, hyaline, branched	Oval conidia, long and cylindrical conidiophores and spherical vesicles	*Aspergillus niger*
AR5	Green, granular and compact colony shape, flat edges and smooth texture	Yellowish white	Non-septa, hyaline, branched	Oval conidia, long and cylindrical conidiophores and spherical vesicles	*Aspergillus flavus*
AR7	Green with grayish-white edging, jagged edges and dense texture	Yellow	Septa, hyaline, branched	Conidia are spherical and vened-septa conidiophores like shrubs	*Penicillium* sp. 2

Table 7: Type of antagonistic interaction of rice rhizosphere fungus against *P. oryzae* haplotype C–011

	Types of antagonistic interactions		
Dual culture	Competition	Antibiosis	Parasitism
Trichoderma sp.+*P. oryzae*	√	-*	√
Paecilomyces sp.+*P. oryzae*	-	√	-
Penicillium sp.1+*P. oryzae*	√	-*	√
Aspergillus niger+*P. oryzae*	√	√	-
Aspergillus flavus+*P. oryzae*	√	√	-
Penicillium sp 2+*P. oryzae*	-	√	-

-: No antagonistic interaction, √: Antagonistic interactions and *Antibiotic ability

Table 8: Type of antagonistic interaction of rice rhizosphere fungi against *P. oryzae* haplotype D–111

	Types of antagonistic interaction		
Dual culture	Competition	Antibiosis	Parasitism
Trichoderma sp.+*P. oryzae*	√	-*	√
Paecilomyces sp.+*P. oryzae*	-	√	-
Penicillium sp. 1+*P. oryzae*	√	-*	√
Aspergillus niger+*P. oryzae*	√	√	-
Aspergillus flavus+*P. oryzae*	√	√	-
Penicillium sp. 2+*P. oryzae*	-	√	-

-: No antagonistic interaction, √: Antagonistic interactions and *Antibiotic ability

Table 9: Type of antagonistic interaction of rice rhizosphere fungus against *P. oryzae* haplotype–F110

	Types of antagonistic interaction		
Dual culture	Competition	Antibiosis	Parasitism
Trichoderma sp.+*P. oryzae*	√	-*	√
Paecilomyces sp.+*P. oryzae*	-	√	-
Penicillium sp. 1+*P. oryzae*	√	-*	√
Aspergillus niger+*P. oryzae*	√	√	-
Aspergillus flavus+*P. oryzae*	√	√	-
Penicillium sp. 2+*P. oryzae*	-	√	-

-: No antagonist interaction, √: Antagonist interaction and *Antibiotic ability

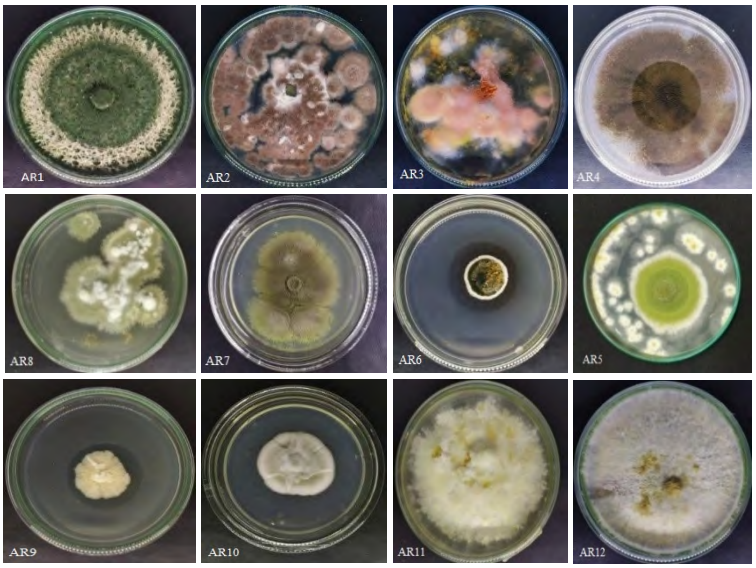

Fig. 1: Pure cultures of 12 rice rhizosphere fungal isolates

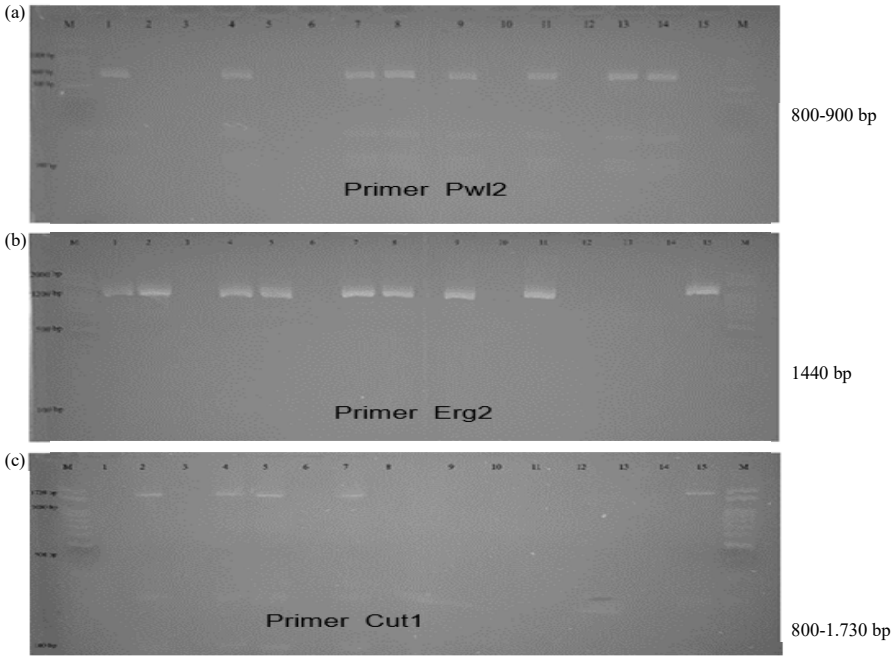

Fig. 2(a-c): Amplification of *P. oryzae* genomic DNA using virulence gene specific primers, (a) Pwl2, (b) Erg2 and (c) Cut1
M: DNA ladder and 1-15: *P. oryzae* isolates

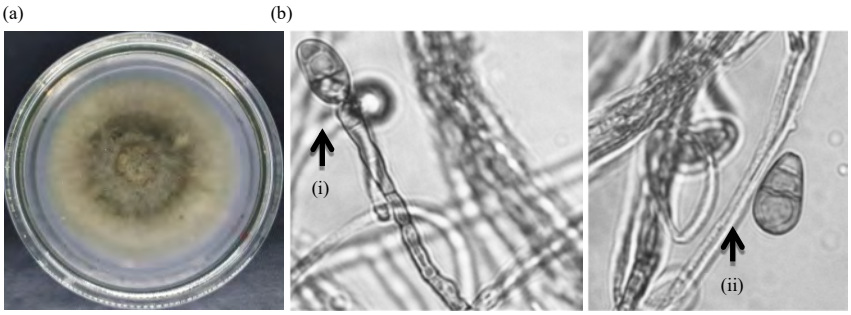

Fig. 3(a-b): *Pyricularia oryzae* haplotype C-011, (a) Macroscopic and (b) Microscopic
(i) Conidia still attached to conidiophore with two septa, (ii) Conidia with two septa, 40x microscope magnification

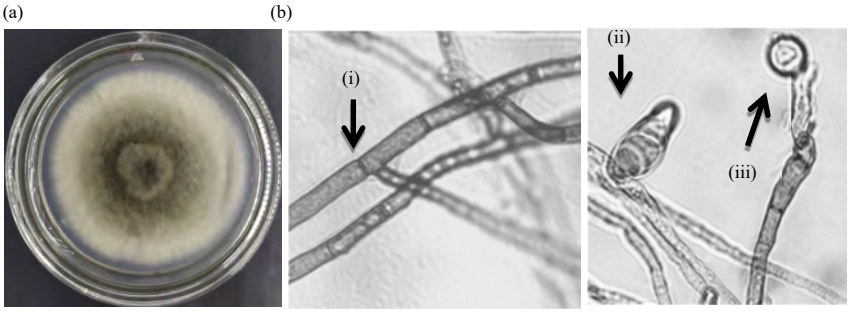

Fig. 4(a-b): *Pyricularia oryzae* haplotype D-111, (a) Macroscopic and (b) Microscopic
(i) Hyphae with septa, (ii) Conidia with two septa, (iii) Chlamydospores and 40x microscope magnification

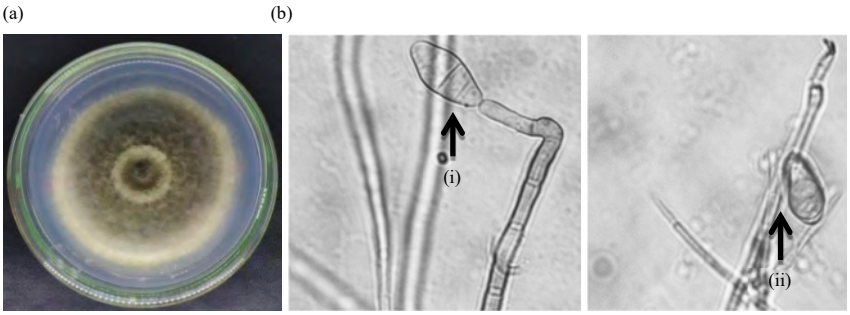

Fig. 5(a-b): *Pyricularia oryzae* haplotype F-110, (a) Macroscopic and (b) Microscopic
(i) Conidia are still attached to the conidiophore with two septa, (ii) Conidia with two septa and 40x microscope magnification

Antagonism test of rhizosphere fungi against three *P. oryzae* haplotypes: Isolates of *Trichoderma* sp. and *Penicillium* sp. 1 quickly filled the surface of the media and pathogen, while isolates of *Paecilomyces* sp., *Aspergillus niger* and *A. flavus* easily spread quickly on the media but did not cover the surface of the colonies of *P. oryzae* haplotypes C-011, D-111 and F-110, then *Penicillium* sp., 2 can grow and spread on the media more slowly than the other antagonistic fungal isolates (Fig. 6-8). *In vitro*, antagonistic tests at 6 and 12 dai inhibited *P. oryzae* haplotypes C-011, D-111 and F-110. Observations showed antagonistic interactions, namely competition, antibiosis and parasitism in rhizosphere fungi against *P. oryzae* in each haplotype tested (Fig. 6-8). Isolates of *Trichoderma* sp. and *Penicillium* sp., 1 showed the interaction of space competition and hyperparasitic rhizosphere fungi against *P. oryzae* haplotypes C-011, D-111 and F-110. In the *Paecilomyces* sp., isolate, there was a purplish color on the side of the meeting with the pathogen colony, while in the *Aspergillus niger*, *A. flavus* and *Penicillium* sp., 2 isolates, there was a yellow color on the side

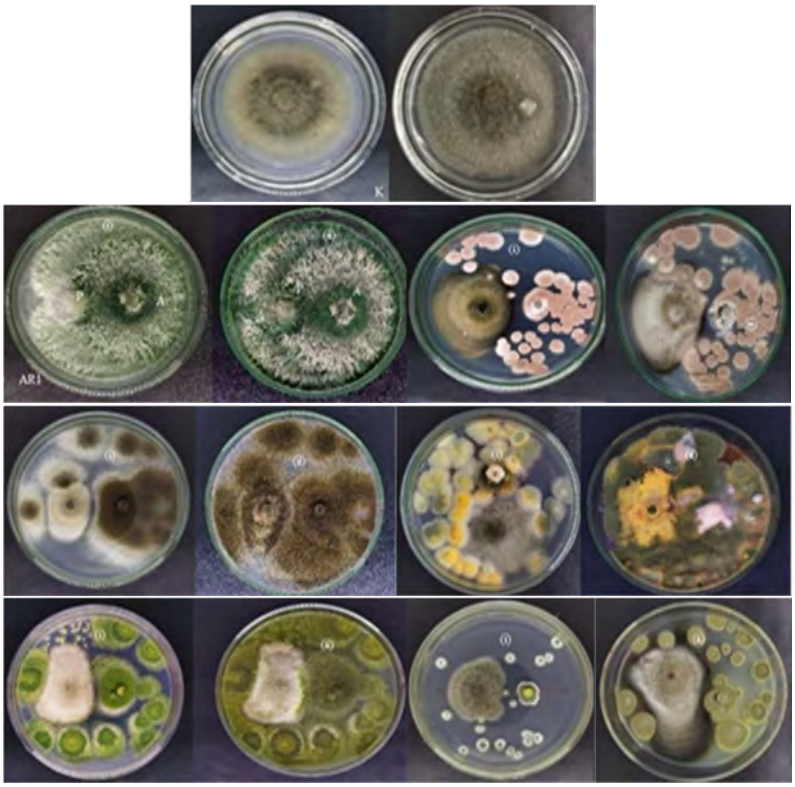

Fig. 6: Dual culture of rhizosphere fungi and *P. oryzae* haplotype C-011
(i) 6 hsi, (ii) 12 hsi, (K) Control, (P) Pathogen, (A) Antagonistic fungi, (AR1) *Trichoderma* sp., (AR2) *Paecilomyces* sp., (AR3) *Penicillium* sp. 1, (AR4) *Aspergillus niger*, (AR5) *A. flavus* and (AR7) *Penicillium* sp. 2

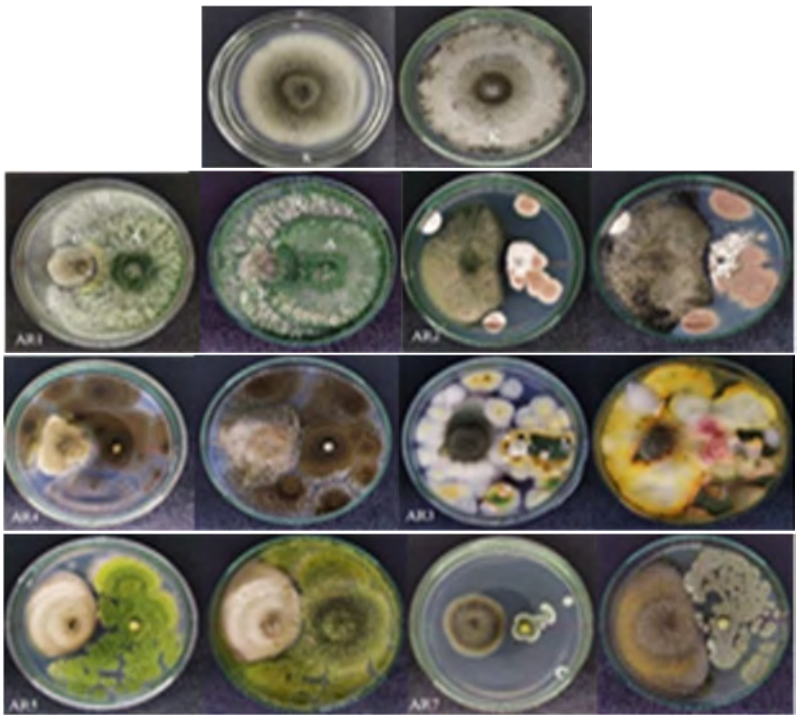

Fig. 7: Dual culture of rhizosphere fungi and *P. oryzae* haplotype D-111
(i) 6 dai, (ii) 12 dai, (K) Control, (P) Pathogen, (A) Antagonistic fungi, (AR1) *Trichoderma* sp., (AR2) *Paecilomyces* sp., (AR3) *Penicillium* sp. 1, (AR4) *Aspergillus niger*, (AR5) *A. flavus* and (AR7) *Penicillium* sp. 2

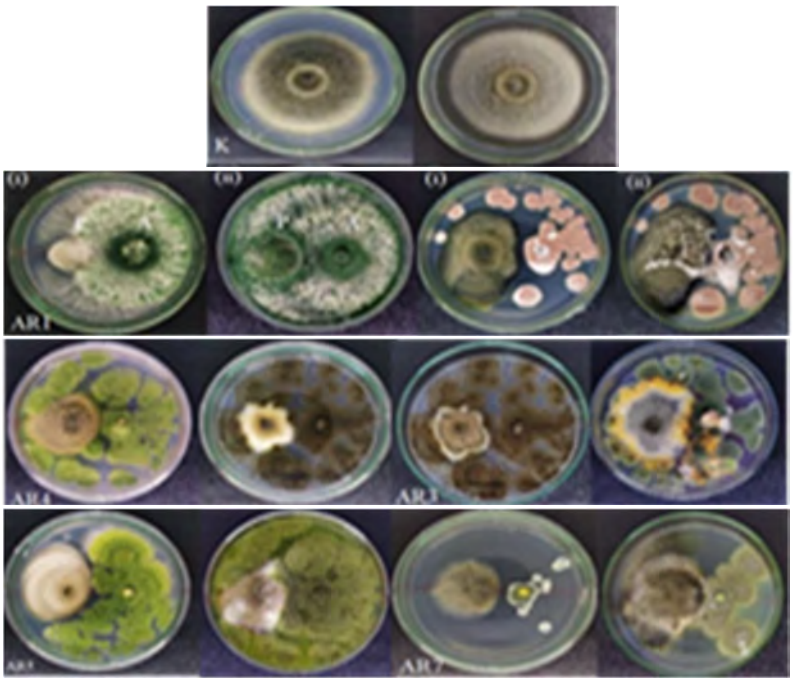

Fig. 8: Dual culture of rhizosphere fungi and *P. oryzae* haplotype F-110
(i) 6 hsi, (ii) 12 hsi, (K) Control, (P) Pathogen, (A) Antagonistic fungi, (AR1) *Trichoderma* sp., (AR2) *Paecilomyces* sp., (AR3) *Penicillium* sp. 1, (AR4) *Aspergillus niger*, (AR5) *A. flavus* and (AR7) *Penicillium* sp. 2

of the meeting with the pathogen colony. The color formed on the side of the meeting of the tested rhizosphere fungal colonies antagonistic to the pathogen colonies indicates that the tested rhizosphere fungal isolates have antibiosis ability (Fig. 6-8). The following are the types of interactions of antagonistic fungi with *P. oryzae* haplotypes C-011, D-111 and F-110 (Table 7-9).

DISCUSSION

The results of the exploration of rice rhizosphere fungi obtained 12 isolates of antagonist candidates that have macroscopic differences including color, colony surface and texture. The differences in these characteristics indicate that in the rhizosphere area of local aromatic rice plantations of Remaja and Bandarata variety, there are many types of fungi that interact. The diversity of soil fungal communities is related to the diversity of above-ground plant communities that contribute to improving rhizosphere soil health and pathogen control[28]. Various types of rhizosphere microbial roles include plant growth-promoting fungi (PGPF), can produce defense enzymes, antibiotics, volatile compounds and phytohormones can be used as a component of plant disease management and can even induce long-term resistance and resistance in plants against pathogens[29]. Pathogen as a control of pathogenic fungi[30]. Soil microbial communities as ecological indicators to evaluate soil health[31].

The morphology of the *P. oryzae* haplotypes (C-011, D-111 and F-110) tested had colony colors that varied from grey to blackish and produced smooth and rough colony margins. However, there were no variations associated with conidia. Conidia pyriform hyaline to pale olive, 2 septa with 3 chambers. *Pyricularia oryzae* isolates have a colony color that quickly changes from white to dark or vice versa during repeated subculturing in the laboratory. The mycelium of *P. oryzae* is branched and hyaline. The simple stratified conidiophores are sparsely branched which are moderately long and sceptate. Conidia attached sympodially at the end of the conidiophores are generally pyriform to obclavate. Blast fungus isolates in rice tend to be unstable in colony appearance, fertility and pathogenicity during repeated subcultures in the laboratory[13].

Agroecological differences in the source of *P. oryzae* isolates can lead to great differentiation in genomic DNA. The environment can affect inoculum availability, pathogen growth rate, pathogen survival and host genetic susceptibility as well as the direction and distance of pathogen spread. Isolates containing 1 or 2 unstable virulent genes may often undergo spontaneous mutations that have a high effect on virulence[21]. *Pyricularia oryzae* having genes with normal mutation frequencies suggests that genetic instability can affect specific parts of the *P. oryzae* genome and the effects vary by race.

The ability of rhizosphere fungal isolates to inhibit the growth of *P. oryzae* haplotype C-011 (Table 3). Treatment AR1 had 90% inhibition and AR3 obtained 82% inhibition so that it was categorized as potential inhibition of radial growth (PIRG) very high and reached a ball rating score of 1, meaning that the antagonist exceeded the pathogen boundary and covered the entire surface of the media. Treatment AR2 obtained 60% inhibition, AR4 obtained 73% inhibition, AR5 had 71% inhibition and AR7 had 63% inhibition, it was categorized in high PIRG with a uniform ball rating score of 2, meaning that the antagonist agent controlled two-thirds of the media surface. The ability of rhizosphere fungal isolates to inhibit the growth of *P. oryzae* D-111 (Table 4), the high PIRG category is obtained in treatment AR1 has 72% inhibition, AR3 has 62% inhibition, AR4 has 65% inhibition and AR5 has 64% inhibition and reaches a uniform ball rating score of 2, indicating that the antagonist agent controls 67% of the media surface.

The inhibition of treatments AR2 51% and AR7 55% with a medium PIRG category and achieved a bell rating score of 3, meaning that each antagonist and pathogen occupied 50% of the media surface and no other organisms dominated. The inhibition of rhizosphere fungal isolates against the growth of *P. oryzae* haplotype F-110 (Table 5). Treatment AR 1 had 83% inhibition with a very high PIRG category and achieved a ball rating score of 1, meaning that the antagonistic agent completely surpassed the pathogen and covered the entire surface of the media. Treatments AR2 and AR5 had 55% inhibition and AR7 obtained 53% inhibition with a medium PIRG category and achieved a bell rating score of 3, meaning that each antagonist and pathogen occupied 50% of the media surface and no other organisms dominated. The inhibition power of AR3 is 73% and AR4 63% so it falls into the high PIRG category and reaches a ball rating score of 2, meaning that the antagonist controls 67% of the media surface. Mutation and recombination are the main sources for plant pathogenic fungi to produce genetic variation. Isolates containing one or two unstable virulent genes such as F-110 are likely to undergo frequent spontaneous mutations that affect their virulence[21]. *Pyricularia oryzae* isolates containing one or more unstable virulent genes such as haplotypes D-111, F-101 and G-100[13].

The results of macroscopic and microscopic identification of AR1 isolates are *Trichoderma* sp., fungi, appearing velvety thick smooth mycelium, radial colony growth with a clear ring pattern, green and white colonies, round conidia and grow at the end of conidiophores and have short phialid stalks. Conidiophores of *Trichoderma* sp., fungi are hyaline in color, branched, single phialide on each branch have round conidia and have rapid growth[20]. The microscopic appearance of *Trichoderma* sp., is green hyphae, short phialid stalks, greenish conidia in the form of globules (round) growing at the ends and there are also conidiums formed in clusters of light green on the surface of the conidiophore cells. Many branching conidiophores resemble pyramids, namely longer branches below, phialides were arranged in different groups and there were 2-3 phialids per group[32]. Biocontrol mechanism of *Trichoderma* were antibiosis, competition, and mycoparasitic, *Trichoderma* biocontrol mechanisms are antibiosis, competition and mycoparasitic. Stimulation of each process involves the biosynthesis of targeted metabolites such as growth regulators, enzymes, siderophores and antibiotics[33]. *Trichoderma koningiopsis* Cachara significantly reduced *P. oryzae* mycelial growth *in vitro* and blast disease severity in wheat plants[34].

Isolate AR2 is *Paecilomyces* sp., has fast colony growth development, woolly, powdery or powdery texture, golden, yellowish-green color, sometimes yellow-brown, purple or tan, never green or blue-green as in *Penicillium* sp. It has a swollen phialide at the base of the colony, gradually tapering to a rather long and slightly slender neck and occurs solitarily in paired colonies, as verticils on the penicillate head. Conidia are long chain-like, single-celled, hyaline, ovoid to fusoid conidia produced in basipetal succession from the phialide. *Paecilomyces* sp., have hyaline and septate hyphae. Branching of the conidiophores occurs on rings like petioles or flowers at several levels. Conidia measure 2-13 µm in length and have a single cell that is oval to pyriform, some are uninstaller and some are chained. *Paecilomyces* sp., is a genus of the order Eurotiales, phylum Ascomycota. The genus *Paecilomyces* sp., can be distinguished from the genus *Penicillium* sp. Although it has a close relationship with one phylum, the difference is that it has longer and slender phialides.

The form of *Paecilomyces* sp., inhibition of the pathogen is antibiosis, which is indicated by the antagonistic interaction type of the fungus against 3 haplotypes of *P. oryzae*. The potential to inhibit other fungi can be utilized as a biological agent. The genus *Paecilomyces* is a cosmopolitan fungus described as a biostimulant of plant growth and crop yield and as a biological agent for plant disease control[35]. *Paecilomyces* metabolite production contributes to bioleaching[36]. *Paecilomyces* variety has been shown to produce a lot of siderophores[37].

Isolates AR3 and AR7 resembled *Penicillium* sp., fungi, having erect conidiophores and branching circularly both single and double and resembling the branching shape of bushes. Conidia are produced at the end in a series, round in shape. Conidiophores are fingered and there are 2-3 branching hyphae[20]. *Penicillium* is a filamentous fungus that can produce bioactive compounds including pigments. *Penicillium* sp. (GEU_37) isolated from soil is a strain that can sporulate, exudate and diffuse higher red pigments in potato dextrose[38]. *Penicillium* sp., showed antifungal activity based on inhibition of pathogen mycelial growth. *Penicillium* sp., CH6 is a microbe that can be explored as an alternative microbe to protect fruits from diseases caused by fungi[39]. *Penicillium* sp., the CRM 1540 has been isolated and identified as capable of producing penicillin acid, which can be an alternative against citrus fruit rot. *Penicillium linzhiense* is a new species isolated from soil. The comes about appeared tall antifungal movement against *P. oryzae* that causes impact in rice with an hindrance rate of up to 77%, whereas for three other citrus pathogens, *Diaporthe citri*, *Phyllosticta citrichinaensis* and *Colletotrichum gloeosporioides*, the hindrance rates were 40, 50 and 55%, respectively. Subsequently, it has the potential to be utilized as a biocontrol specialist, particularly for modern pathogens on citrus plants[40].

Fungal isolates AR4 and AR5 have similar characteristics to *Aspergillus* sp. Macroscopically, the mycelium appears smooth and thin like cotton and the edges of the colonies are flat. Spores are obvious on the surface of outspread colonies, conidia are circular, vesicles are circular and conidiophores are long and round and hollow, colonies and spores are blackish brown with white edges and dark spores show up in confine AR4 taking after *A. niger* whereas green colonies and spores with white colony edges show up in separate AR5 taking after *A. flavus*.

Aspergillus niger is characterized by blackish-white spores and the color intensity increases in older cultures. The shape of the colony surface is raised with a smooth texture on the PDA medium. *Aspergillus niger* has microscopic characteristics of round-shaped vesicles with diameters ranging from 17.52 to 23.4 µm. On the surface of the vesicle, there is a sterigma then fialid, the conidia are round with a diameter range between 3.5 to 4.5 µm. The conidiophores are long and cylindrical and colorless (hyaline)[41]. *Aspergillus flavus* has a green to yellowish-green colony morphology with a granular and compact colony shape. Young colonies are white and the colour turns yellowish green after forming conidia. Observations of *A. flavus* showed vesicles that were round to oval in shape with a diameter of 25-45 µm. The conidia are circular and 3-6 µm in distance across and the conidiophores are long and round and hollow. *Aspergillus flavus* has green colonies with white colony edges, encompasses a smooth or wooly colony surface with a floccose center, the conidiophores are colorless, thick-walled, unpleasant and have pad vesicles, the vesicle shape is circular to sub-globose, the metal covers the surface of the vesicle and is in all bearings, the conidia are circular, thin-walled and marginally unpleasant[42].

Soil fungi belonging to the genus *Aspergillus*, namely *A. niger* and *A. flavus* as the most important species. *Aspergillus* spp., isolated from healthy paddy fields were able to inhibit *P. oryzae* with an optimum inhibition percentage of 100% at all concentrations of secondary metabolites aged 14 days[43]. Non-aflatoxigenic *A. flavus* (AF) strains as biocontrol agents can control AF contamination in cereal crops up to 99.3%[42].

CONCLUSION

Twelve fungal isolates from the rhizosphere of aromatic rice were successfully isolated and six antagonistic fungal isolates were able to inhibit the growth of *P. oryzae* haplotypes C-011, D-111 and F-110. *Trichoderma* sp., isolates had the highest inhibition percentage of 72-90%, followed by *Penicillium* sp., 1 with an inhibition percentage of 62-82%. *Trichoderma* sp. and *Penicillium* sp., 1 have the potential as biological control agents to inhibit the fungus *P. oryzae* that causes blast disease in rice plants.

SIGNIFICANCE STATEMENT

This study has isolated and collected several antagonistic fungi from the local aromatic rice rhizosphere and can be used as a reference for controlling blast disease. This study will help researchers determine the genus of the fungus through morphological identification, determining the type of antagonistic fungus interaction with the 3 haplotypes of *P. oryzae*. The results of this research provide a fundamental contribution in the field of *P. oryzae* control which will become a reference in environmentally friendly control to realize a sustainable rice crop management system.

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