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Characterization of the Sheath Blight Complex of Fungi in Rice (*Oryza sativa* L.)

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RICE sheath blight is the most economically substantial rice disease worldwide that leads to significant grain yield and quality losses. *Rhizoctonia solani* is the causal agent of sheath blight. However, other fungal species are also associated with sheath blight and could aggravate the disease complex. This study investigated the fungi associated with the sheath blight complex of rice (*Oryza sativa* L.). Three isolates showed different morphological characteristics in terms of colony color, hyphal size, hyphal branching, and sclerotia formation and size. Identification by Internal Transcribed Spacer (ITS) sequence revealed their identities as *R. solani* AG1-IA, *Chaetomium globosum*, and *Poitrasia circinans*. *C. globosum* and *P. circinans* were associated with rice sheath blight. A phylogenetic tree differentiated *R. solani* AG1-IA from *C. globosum* and *P. circinans* and revealed other *R. solani* AG-4 and AG2-2IIIB subgroups and fungi that could cause the sheath rot of rice (*Fusarium proliferatum, F. hainanense, F. sulawesianense*, and *Sarocladium oryzae)*.

Keywords: Characterization, Internal Transcribed Spacer, Rice, *Rhizoctonia*, Sheath blight.

Introduction

Rice sheath blight is the most economically significant rice disease worldwide, particularly in tropical Asia and southern United States. This disease occurs in warm and humid areas, conditions favored by the pathogen, and causes yield losses ranging 4%–50% depending on the crop stage at the time of infection, disease severity, and environmental conditions (Singh et al., 2016). The causal agent of sheath blight in rice is *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk]. *R. solani* has 14 anastomosis groups, namely, AG1 to AG13 and AGBI. Group AG1 has subgroup AG1-IA, which is the most important group of pathogens causing sheath blight, banded leaf, aerial blight, and brown patch in more than 27 families of dicots and monocots (Singh et al., 2016). *R. solani* can be identified through morphological observation (Kumar et al., 2008; Lal & Kandhari, 2009; Moni et al., 2016), ITS rDNA sequencing (Toda et al., 1999; Pascual et al., 2000; Priyatmojo et al., 2001, Toda et al., 2004; Bintang et al., 2017), or specific primers for various subgroups (Salazar et al., 2000; Sayler & Yang, 2007; Fredricks et al., 2010). Rice sheath blight is caused by *R. solani* and two other *Rhizoctonia* viz. *R. oryzae* and *R. oryzae sativae,* which are linked to the sheath blight complex; among which, *R. solani* is the most virulent (Hu et al., 2010). Sheath blight complex has also been linked to *Corticium sasakii* (Shirai) Matsumoto, *C. vagum* Berk. & Curt., *Sclerotium irregulare* I. Miyake, *Hypochnus sasakii* Shirai, and *Pellicularia sasakii* (Dhirau) S. Ito I. (Lore et al., 2015). This study aimed to characterize the fungi associated with the sheath blight complex of rice (*Oryza sativa* L.) based on their morphological and molecular characteristics.

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Materials and Methods

Sample collection and culture

Rice midribs showing sheath blight symptoms were collected from local variety Menor from the rice field of West Progo Regency, Special Region of Yogyakarta, Indonesia. Isolation was conducted by cutting the samples into small pieces, sterilizing with sodium hypochlorite (1%) solution, and washing with sterile distilled water. The culture was grown on potato dextrose agar media and incubated at 27°C for 7 days for further observation.

Morphological Characterization

The mycelia of 7-day cultures were mounted on a glycerol-dripped slide with trypan blue or safranine staining. The slides were covered and observed under a microscope. Morphological characterization was conducted to observe colony color, hyphal color, hyphal branching and size, and sclerotia formation and size (Kumar et al., 2008; Lal & Kandhari, 2009; Moni et al., 2016).

Molecular identification

The fungal isolates were identified using universal primers (ITS1/ITS4) and specific primers of *R. solani*. Isolates were grown on PDA and incubated for 7 days. CTAB method was used to isolate the fungal DNA. The ITS 1 and ITS 4 primers (Toda et al., 1999; Pascual et al., 2000; Priyatmojo et al., 2001, Toda et al., 2004; Bintang et al., 2017) were used to amplify the ITS rDNA region. The following primers were also used to detect the specific subgroup of *R. solani*: Rs1F-Rs2R for *R. solani* AG1-IA (Sayler & Yang, 2007), AG2sp-5,8SKhotR for *R. solani* AG2, and AG22sp2-5,8SKhotR for *R. solani* AG2-2IIIB (Salazar et al., 2000, Fredricks et al., 2010). Amplification was carried out using PCR reaction mixture following the method of Kasiamdari et al. (2002). The obtained DNA sequences were analyzed for significant matches with basic sequence alignment BLAST program from NCBI. A phylogenetic tree was constructed for the aligned datasets using the neighbor-joining method (kimura-2 substitution model; gaps treatment as pairwise deletion) in MEGA 6 with 1000 bootstraps.

Results

Morphological characteristics

Plants showing sheath blight symptoms were collected (Fig. 1A), and the sclerotia were observed (Fig. 1J). Three different fungal isolates were isolated and showed different variations in culture characteristics. The MK isolate was characterized as light brown colony, fluffy, hyphal length of 30.06–69.69µm, hyphal width of 5.6–6.9µm, septate, 90° angle of hyphal branch, constriction of hyphae and formation of septa at a short distance from the point of the hyphal branches. The sclerotia were light brown, scattered aerially and on the surface, and rough with a diameter of 1–2mm (Figs. 1B, 1E, 1I). The MS isolate was characterized as light brown colony, cottony, hyphal length of 15.69–49.57 µm, hyphal width 1.73–5.07 µm, septate and branched hyphae, and no sclerotia (Figs. 1C and 1F). The MG isolate was characterized as whitish brown colony, cottony, hyphal length of 36.14–223.14 µm, hyphal width of 7.27–9.55 µm, aseptate and branched hyphae, and no sclerotia (Figs. 1D and 1G).

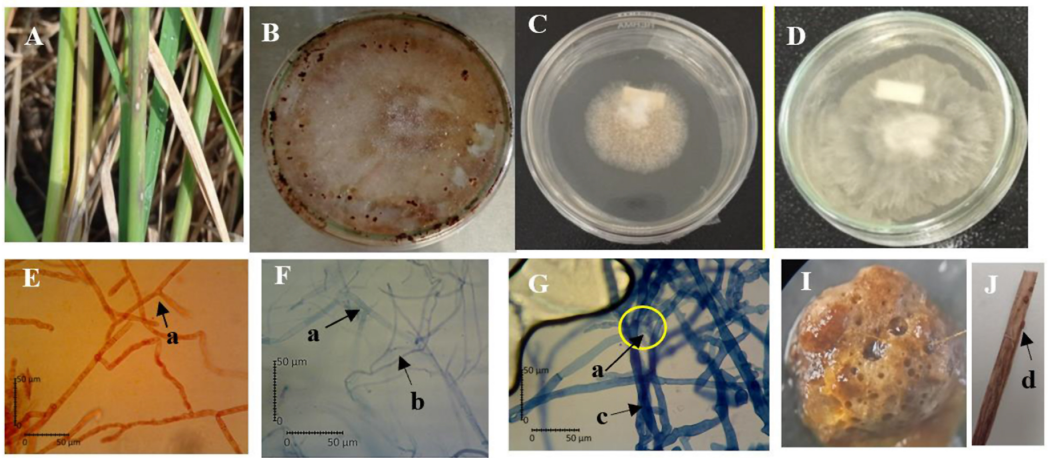

Fig. 1. Symptom of sheath blight on rice (A) and sclerotia (J). Colony culture of fungal isolates of MK (B), MS (C), and MG (D) [E= Hyphae of MK isolate (a), F= Hyphae of MK isolate (a) and MS isolate (b), G= Hyphae of MK isolate (a) and MG isolate (c), and I= Sclerotia of MK isolate]

On the basis of the morphological characteristics, the MK isolate had the following similar characteristics to *R. solani*: hyphae branching at a 90° angle, constriction of hyphae, formation of septa at a short distance from the point of the hyphal branches' origins, and production of sclerotia. The two other fungal isolates (MS and MG) did not show the morphological characteristics of *Rhizoctonia* (Figs. 1C and 1D).

Molecular identification

Detection of fungi by specific primer

Molecular detection using specific primers Rs1F/Rs2R showed the positive amplification (140bp) of MK isolate with strong band (Fig. 2), indicating the identity of *R. solani* AG1-IA. For MS and MG isolates, positive amplification was observed with weak bands of the same size (140bp) (Fig. 2)..

Sequence analysis of rDNA-ITS region

ITS rDNA sequence with ITS1/ITS4 primers produced 631bp of ITS1, 5.8S rDNA, and ITS2 region. Nucleotide sequence analysis of ITS rDNA using BLAST homology search showed that the MK isolate had 99.86% identity with *R. solani* isolate RUP02 from Malaysia (KX674518). The ITS rDNA sequence of the MS isolate (528bp) showed 99.83% identity with *Chaetomium globosum* isolate A2S2-D10 (KJ767122) from Penang, and that of the MG isolate (626bp) had 99.66% identity with *Poitrasia circinans* isolate INF8 from India (MZ227286) (Table 1).

Phylogenetic tree

Phylogenetic analysis showed that the MK isolate was identified as *R. solani* AG1-IA and was clustered with other *R. solani* AG1-IA members, namely, KF053535 (*R. solani* isolate GDHZ12, 99.86% sequence similarity), MK213724 (*R. solani* AG1-IA, 99.44% sequence similarity), FJ236314 (*Thanatephorus cucumeris* strain YN3–AG1-IA, 99.58% sequence similarity), and KX674618 (*R. solani* AG1-IA isolate RUP02, 99.58% sequence similarity) but not with *R. solani* AG-4 (MK478913, MK481003) and AG2-IIIB (MZ399250, KR736348). The MG isolate was clustered with *P. circinans* KT277656 with 100% sequence similarity and with M2227286 and MH857733 with 99.66% and 99.65% sequence similarities, respectively. The MS isolate was clustered with *C. globosum* KJ767122 with 99.83% sequence similarity and with MN173145 and KM030576 both with 99.65% sequence similarity. The ITS rDNA sequences of the three isolates (MK, MS, and MK) were different from those of *Sarocladium oryzae* from India (NR145045) and Indonesia (MT012231) associated with sheath rot disease in rice, *Fusarium proliferatum* (MT394055) from India, and *F. hainanense* (MT138475) and *F. sulawesiense* (MT138457) from Indonesia (Fig. 3).

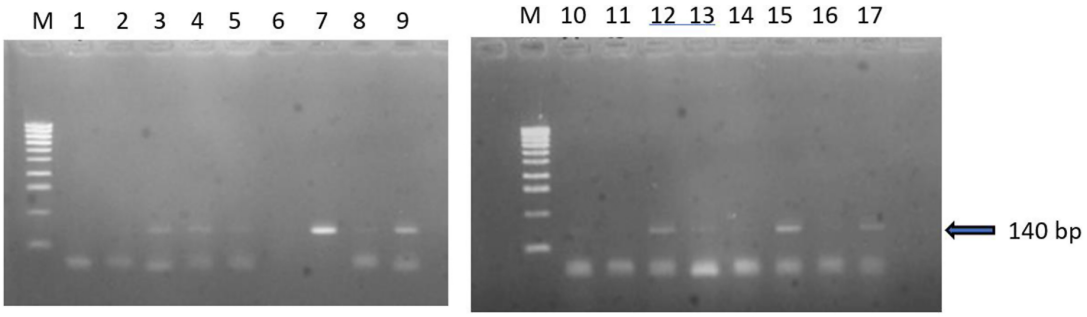

Fig. 2. Specific detection of AG 1-IA isolates of *Rhizoctonia solani* with Rs1F/Rs2R primers Lane 7. *R. solani* isolate MK, Line 8: MS isolate, Line 13: MG isolate. Other lanes showed positive and negative amplifications from other fungal samples isolated from rice fields (Line 1–6, 9–12, 14–17) [M= molecular marker (100bp)]

TABLE 1. Homology search of fungal isolates associated with rice sheath blight using the available sequences from NCBI

No	Code/bp	Accession Number	Species	Host	Query cover	Percent identity	Country origin
1	MK/631	KX674518	*Rhizoctonia solani* AG-1 IA isolate RUP02	-	99	99.58	Malaysia
2	MS/528	KJ767122	*Chaetomium globosum isolate A2S2-D10*	Beach Soil	100	99.83	Penang
3	MG/626	MZ227286	*Poitrasia circinans* isolate INF8	Soil	90	99.66	India

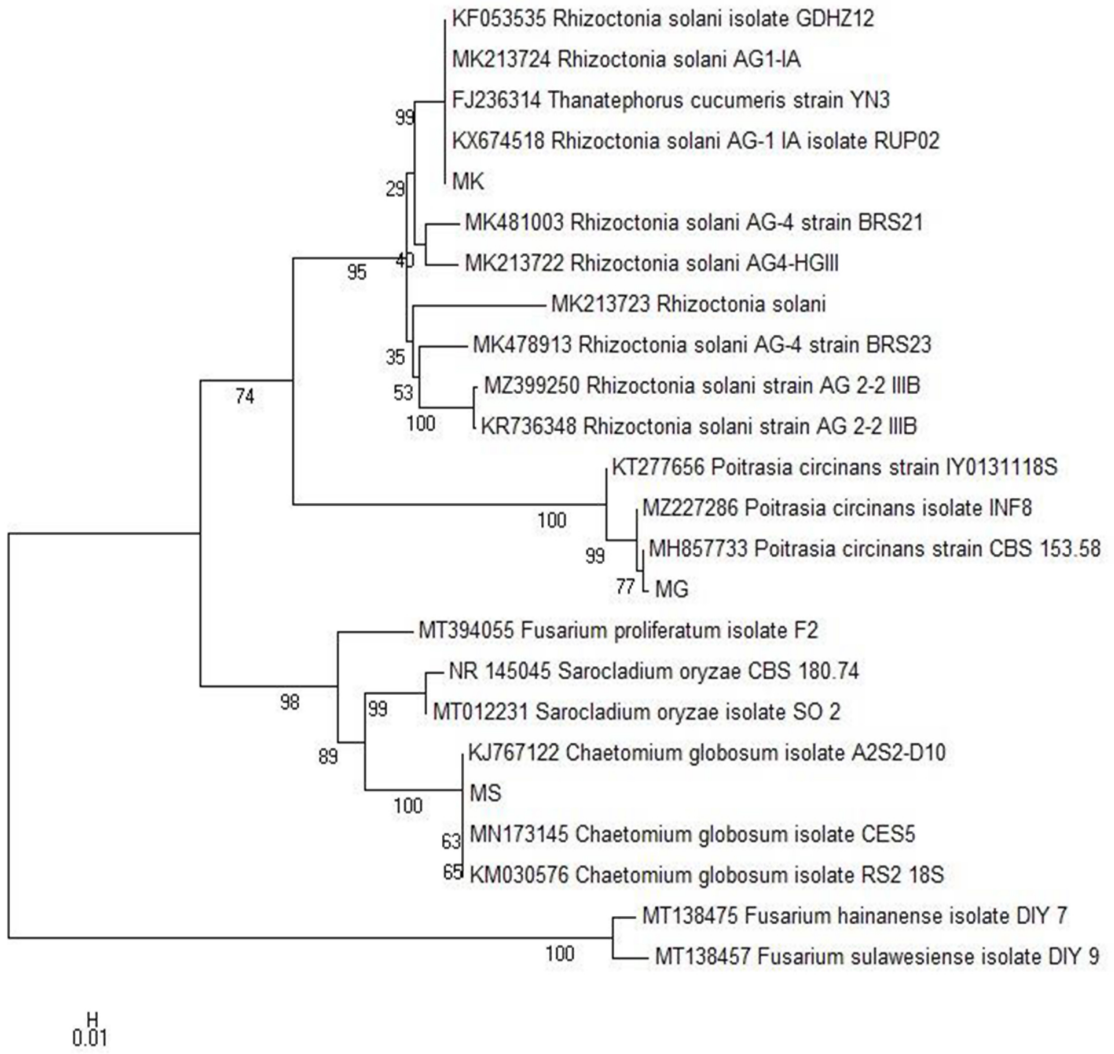

Fig. 3. Phylogenetic tree generated using the neighbor-joining method showing the genetic relationship among the three isolates (MK, MS, and MG) and their association with rice sheath blight and other fungal isolates available in NCBI

Discussion

On the basis of the morphological observation of the colony, the MK isolate produced the typical branching pattern of *R. solani* with the following characteristics: hyphal branching at a 90° angle, constriction at the point of branching of the mycelium, and the presence of a septum near the branching junction as a taxonomical importance of characterization (Moni et al., 2016; Harvianti & Kasiamdari, 2021). Light brown colony, fluffy texture, and sclerotia scattered with a diameter of >1.9mm are also the characteristics of *R. solani* AG1 (Ganeshamoorthi & Dubey, 2015). The two other isolates, MS and MK, did not produce the typical *R. solani* branching pattern and therefore were verified as different fungal species.

Rs1F dan Rs2R primer pair was used for the detection of *R. solani* AG1-IA produced DNA amplification of the MK isolate (thick and clear DNA bands) and for the MS and MG isolates (thin/weak bands). The Rs1F dan Rs2R primers developed by Sayler & Yang (2007) from the ITS region of rDNA are specific to *R. solani* and could detect as low as 1 pg DNA. In this work, the specific primers were not tested for specificity against other fungi; hence, the positive results with weak/thin bands on MS and MG isolates were probably caused by the mixed fungal cultures containing *R. solani* (MK isolate). The Rs1F dan Rs2R primer pair reported

by Sayler & Yang (2007) can sensitively detect small quantities of *R. solani* fungal hyphae DNA. The three isolates had 44%–60% similarity of ITS sequences and did not produce DNA amplification using AG2sp/5,8SKhotR primers for *R. solani* AG2 and AG22sp2/5,8SKhotR primers *R. solani* AG2-2IIIB, indicating that they cannot be classified as *R. solani* AG2 or AG2-2IIIB.

ITS rDNA region analysis is valuable for the identification of AGs and AG subgroups of *R. solani*. Kuninaga et al. (1996) found that the same subgroups have above 96% sequence homology of ITS regions. Isolates within an AG with different subgroups showed 66%–100% sequence similarities, and isolates of different AGs showed 55%–99% sequence similarity. In the present study, homology search through NCBI confirmed the identity of the species with above 99% sequence similarity. *C. globosum*, which has been used as an antifungal to eradicate rice blast (Song & Soytong, 2016; Soesanto et al., 2021), was found to be associated with sheath blight in rice. *Chaetomium* extract produces antifungal-expressing metabolites for *Magnaporthe* sp. (Anamorph; *Pyricularia oryzae*) and causes rice blast (Tongon & Soytong, 2015; Song & Soytong, 2018). Ibiam et al. (2009) isolated *C. globosum* associated with rice seeds from storage and paddy fields in Afikpo, Nigeria and found that *C. globosum* plays a role in the germination of rice seeds. *P. circinans* (syn. *Choanephora infundibulifera*) is dioecious and mucoraceous and has been reported to cause the pod rot of *Vigna sinensis* (Kwon et al., 2001). The discovery of *P. circinans* in rice is probably due to the intercropping of rice planting, a type of crop management that involves two or more types of plants on one land at the same time (Suryanto, 2019).

Phylogenetic analysis using ITS rDNA sequences separated *R. solani* isolates into distinct groups corresponding to different AGs. The MK isolate was clustered with *R. solani* AG1-IA which causes different diseases and comes from other hosts. The ITS sequence showed a close relationship with the *R. solani* from India (Suryawanshi et al., 2019), Malaysia (KX674518), and China (Wang et al., 2015), and the *R. solani* that causes the leaf blight of peanut in China (KF053535). Zhou et al. (2009) reported that ITS sequences are valuable for identifying the AG-4 isolate but not for detecting AG2-1 and AG2-2. The phylogenetic tree showed that *R. solani* AG1-IA had a separate cluster with *C. globosum* and *P. circinans*, thus confirming their identity as different fungal species.

Conclusion

The results showed that sheath blight in rice (*Oryza sativa* L.) is not only caused by *R. solani* AG1-IA. Two additional fungi named *C. globosum* and *Poitrasia circinans* were identified and found to be associated with *R. solani* AG1-IA, the rice sheath blight causative agent, but not as causal pathogens.

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Competing interests: The authors report no conflicts of interest regarding this work.

Authors' contributions: A.N. collected and analysed the data, wrote and revised the manuscript. R.S.K. wrote, revised, and finalized the manuscript**.**

Ethics approval: Not applicable.

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