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Isolation and molecular identification of fungi causing stem rot disease in Bali's local legumes

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ABSTRACT. Efforts to improve food security in Indonesia, particularly Bali, need to be supported by improvements in cultivation techniques, including the management of pests and diseases. Meanwhile, legume crops are often attacked by stem rot diseases which potentially decrease production and leads to economic losses. This disease is generally caused by the soil-borne pathogenic fungus *Sclerotium rolfsii* or *Athelia rolfsii*. The macroscopic and microscopic morphologies of these two species are the same and difficult to distinguish, hence, molecular identification is needed to differentiate between the species. Therefore, this study aims to isolate and molecularly identify the fungi causing stem rot disease in local legume plants in Bali. The methods used include isolation of pathogenic fungi from legumes showing symptoms of stem rot disease, pathogenicity test, identification of isolates with the highest virulent levels, DNA extraction, DNA amplification by PCR and electrophoresis, ITS region sequencing and computer analysis sequences. The isolation procedure identified six fungal isolates coded SKT, SKB1, SKB2 SKB3, SKL and SKN isolates. SKT isolates had the highest virulence rate as indicated by the pathogenicity test of peanut plants. Furthermore, molecular identification results show that SKT isolate is *Athelia rolfsii*, a similar clade with the fungi sequences in GenBank with 100% bootstrap support.

Keywords: *Athelia rolfsii*; PCR; phylogeny analysis; *Sclerotium rolfsii*; soybeans and peanuts

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INTRODUCTION

Legume crops are often attacked by stem rot diseases which potentially decrease production and lead to economic losses (Devi *et al*., 2017; Parveen *et al.*, 2017; Parwanayoni *et al*., 2019). According to the BPS (2020), Indonesia has continued to import soybeans for the past 20 years, hence, imported soybeans have dominated food processing of raw materials such as tempe. Meanwhile, stem rot disease in soybean plants occurs more rapidly during the rainy season and causes crop failure. This disease is mainly caused by soil-borne fungi, namely *Sclerotium rolfsii* or *Athelia rolfsii* (Darvin, 2013; Doley & Jite, 2013; Adeleke, 2016). Symptoms of stem rot disease in legume plants include initially drooping of leaves close to the ground, while the branches become wilted and yellowish. Furthermore, the leaves slowly turn dark brown and sometimes fall from the plants prematurely, while the branches wither and also turn dark brown, and the lower trunk adjacent to the ground is covered with white mycelium and sclerotia growth (Karthikeyan *et al*., 2015; Paul *et al*., 2017; Nugroho *et al.*, 2019).

A. rolfsii is the sexual (teleomorphic) form of *S. rolfsii* and this fungus has a defense structure called sclerotia, which last for a long time in the soil. Under suitable environmental factors, sclerotia germinate and infect its host plants (Thiessen & Woodward, 2012; Shen *et al.*, 2020). Furthermore, *S. rolfsii* or *A. rolfsii* survives in the soil in the form of sclerotia, and when a suitable host plant is found, it causes infection of the plant (Parmar *et al.*, 2015; Kwon *et al.*, 2017). These fungi cause major economic losses in agriculture, due to its defense structure, namely sclerotia which survive in the soil for years, thereby, making this pathogen difficult to control (Madhuri & Gayathri, 2014; Guerra *et al*., 2015; Sneha *et al.*, 2016). *A. rolfsii* produces an extracellular enzyme, namely cellobiose dehydrogenase

(CDH) used to penetrate the host plant, while *S. rolfsii* also produce six extracellular enzymes, including amylase, carboxymethyl cellulase, lipase, laccase, catalase and gelatinase which are used to infect plant tissues (Chaurasia *et al*., 2015; Elias *et al*., 2015; Sennoi *et al*., 2021). The macroscopic and microscopic morphology of the two species are the same and difficult to distinguish, hence, molecular identification is needed to differentiate between the species (Paul *et al.*, 2017).

Our survey conducted in 2018, at several legumes planting centers (peanuts and soybeans) in four Bali regencies found that the highest percentage of stem rot diseases occurred in Badung 30%, Klungkung 20%, Buleleng 10%, and 6% in Tabanan Regency. This data shows that stem rot disease in legumes is still high in Bali, therefore, there is need to determine the species causing disease in plants for precise and effective control implementation. Improper control leads to various consequences such as resistance to pathogens and environmental damage, therefore, molecular identification of species is needed to provide definite and accurate results. The species found in this study provides the basis for further research, particularly on the effectiveness of disease control.

MATERIALS AND METHODS

Isolation of pathogenic fungi. The fungi were isolated from the stems and roots of legume plants (soybeans and peanuts) which show symptoms of stem rot disease (Fig. 1). The sampling was carried out at the soybeans and peanuts planting center in four regencies in Bali including Badung, Tabanan, Buleleng and Klungkung. Stems and roots of legume plants showing symptoms of stem rot were first cleaned and rinsed with running and sterile water respectively, cut into 1 cm. The samples were placed in the petri dish containing PDA media and then incubated at 25°C until a fungus colony grew around the pieces of the plant. Furthermore, the fungal colonies produced were purified by transferring to a new PDA media to obtain a pure culture and to observe the colony morphology and color as well as sclerotia size (Henuk *et al*., 2017; Yusnawan *et al*., 2019; Prihatiningsih *et al*., 2020; Sennoi *et al*., 2021).

Fig. 1. Legume plants attacked by stem rot disease.

Pathogenicity test. The pathogenicity test was carried out to obtain the most virulent isolates using polybags filled with planting media. Before inoculating the isolates (pathogens) into the planting media, starch slurry potato was added with 3 g each. Furthermore, the samples were inoculated with pathogen isolates, and the peanut seeds were planted, meanwhile, symptoms of stem rot were observed 3 days after inoculation with the pathogen. The isolate with the fastest ability to cause symptoms of stem rot indicates the highest level of virulence. The isolates were then identified molecularly, to determine the species (Sana *et al.*, 2017; Shen *et al*., 2020).

DNA extraction. DNA extraction was performed by first rejuvenating fungal isolates on PDA media for three days at room temperature (25°C). Hyphae at the edges of the fungal colony were collected, placed into centrifuge bottles, and then suspended with 100 µl PrepMan Ultra Reagents (PrepMan Ultra Protocol, Applied Biosystem, USA). Furthermore, the samples were vortexed for 30 s and then placed in a heat block temperature of 95°C-100°C for 10 min and at room temperature for 2 min. The samples were further centrifuged at 10000 rpm for 2 min, while the pellets were collected as DNA extracts for further analysis. The DNA obtained was used as a template for amplification by the PCR (Polymerase Chain Reaction) method using primary pairs ITS 1 (primary forward) and ITS 4 (reverse primary) (Green & Sambrook 2012).

DNA amplification. DNA amplification was performed on 18S rRNA Genes, with PCR

using the Internal Transcript Spacer primer (ITS) 1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS 4 (5'–TCCTCCGCTTATTGATATGC–3') with 600 bp PCR products. The reaction was carried out using the Takara PCR personal thermal cycler (Takara Bio, Otsu, Japan) with Ex Tag (Takara Bio, Otsu, Japan) under the following conditions; Pre-denaturation 94°C (4 min), 35 cycles of denaturation of 94°C (35 s), annealing 52°C (55 s), elongation 72°C (2 min) and post elongation 72°C for 10 min. Furthermore, the PCR results were visualized in 1% agarose gel and electrophoresed in tris-acetic EDTA buffer (TAE), stained with non-toxic coloring (PeqGREEN). Electrophoresis was carried out at a voltage of 50 volts for 100 min, meanwhile, DNA amplification produced fragments measuring up to 600 bp. The DNA fragments produced were then sequenced to identify the fungus species (Iquebal *et al.*, 2017).

ITS Region Sequencing. DNA sequences were determined using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) and the PE Applied Biosystem Automated DNA Sequencer (model 3130×1, Applied Biosystems) according to the manufacturer's guidelines. The double helix DNA sequence was assembled and analyzed sequentially using genetyx (version 11.0) and genetyx-ATSQ (version 4.0) software (Genetyx, Japan), and compared with a similar sequence, from DDB/EMBL/GenBank via NCBI BLAST program. Furthermore, phylogeny analysis was performed using the MEGA 6.0 program, and the Maximum Parsimony (MP) method with a 1000× bootstrap, while the sequence data stored in a notepad with FASTA format were analyzed using the Blast-WU facility available online (Clustalw) at www.ebi.ac.uk, by comparing the similarities between sequences. The results obtained were then used as basic data to create phylogeny trees using MEGA data facilities (Sufaati *et al.*, 2012; Tamura *et al.*, 2013; Hanafiah *et al.*, 2021).

RESULTS AND DISCUSSION

Isolation of pathogenic fungi. The isolation of pathogens causing stem rot disease in legumes produced six fungal isolates coded SKT, SKB1, SKB2, SKB3, SKL, and SKN. The SKT isolates were obtained from Klungkung Regency, SKB1, SKB2 and SKB3 from Badung Regency, SKL from Buleleng Regency and SKN from Tabanan Regency. Furthermore, the six fungal isolates were tested for pathogenicity and showed great potential for causing stem rot symptoms in peanut plants, but the virulence levels were different. The pathogenicity results are presented in Fig. 2.

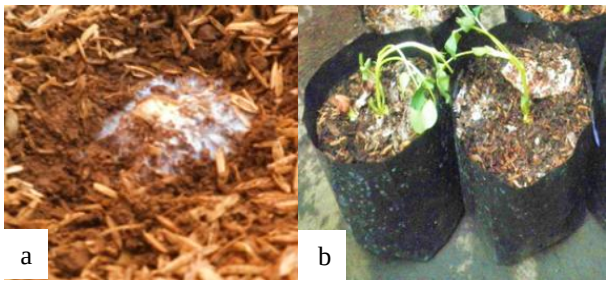

Fig. 2. Pathogenicity test results: a. SKT isolates with the highest virulence levels; b. other isolates.

The pathogenicity test results (Fig. 2) showed that SKT isolate had the highest virulence level, as indicated by the speed of stem rot disease symptoms appearance from the peanut plants, hence, this isolate was used for further testing. Symptoms of the disease appeared about two-four days after inoculation, with symptoms of peanut seeds being overgrown or colonized by fungi, thereby leading to rot and inability to germinate.

Characteristics of SKT isolate fungus. The macroscopic identification results showed that the SKT isolate in PDA media had characteristics such as white colony in the form of a cotton, mycelium with a fan-like structure, growth rate of 9.3 mm/day, thickening and folding of the mycelium after one week, formation of a sclerotia structure which is initially white with soft texture, but gradually turns dark brown with a hard texture. Furthermore, the sclerotia were shown on the PDA media after the isolate was 10-15 days old as presented in a plain view. The sclerotia were round with a diameter of 0.5-1 mm as shown in Fig. 3.

The *S. rolfsii* and *A. rolfsii* fungi have similar colony morphology when grown on PDA media, but both are distinguished by molecular identification (Srividya *et al.*, 2017;

Acabal *et al.*, 2019). These two fungi are synonymous, *A. rolfsii* is a sexual (teleomorphic) form classified into the clas Basidiomycetes, while the asexual form (anamorphic) is *S. rolfsii*, classified into the class Deuteromycetes (Kwon *et al*., 2017). Furthermore, *S. rolfsii* in PDA media show a white feather-like colony and folds at the tip of the mycelia to form sclerotia after 6-10 days of incubation with a diameter of 0.5-2 mm (Daami-Remadi *et al*., 2012; Suheri *et al*., 2014). *S. rolfsii* fungi grown on different media showed different colony diameters during incubation for 72 h, meanwhile, the PDA media produced the largest colony diameter and fastest growth (Muthukumar & Venkatesh, 2013; Mahato & Mondala, 2014). Moreover, the sclerotia diameter also differs, 1.5 and 1.7 mm in the PDA and Oatmeal media respectively (Banakar *et al*., 2017). Thiessen & Woodward (2012) isolated *Sclerotium* from peanuts showing symptoms of stem rot disease and the number of sclerotia as well as sizes were also different. The four isolates showed variations in the size of the colony diameter (ranged from 90.1 to 90.5 mm) and the number of sclerotia. Similarly, the number of sclerotia varied per petri dish with an incubation period of ten days.

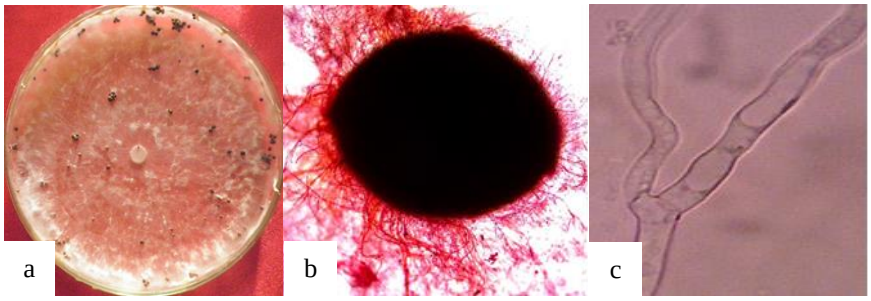

Fig. 3. The microscopic characteristics of SKT isolate: a. SKT isolate fungus on PDA media; b. Germinating sclerotia (magnification 10x); c. Microscopic characteristics of SKT isolate fungi: 1= branched hyphae, 2= septate hyphae (magnification 100).

The microscopic characteristics of SKT isolate including hyphal and sclerotia features observed using a light microscope (Fig. 3). These isolates have cylindrical hyphae and septa, as well as branched hyphae and clamp connections. Meanwhile, clamp connection in hyphae was observed microscopically after incubation for five days on PDA media. Ünal *et al*. (2017) stated that *S. rolfsii* has branched hyphae, clamp connection with a size of 10 µm, and sclerotia on the PDA media after 10 days of incubation with a diameter of 1-3 mm. Furthermore, Zhou *et al*. (2014) observed that *A. rolfsii* had branched and insulated hyphae, while Nugroho *et al.* (2019) reported that the hyphae diameter of *S. rolfsii* isolates from *Cucumis melo* plants with symptoms of stem rot disease, ranged from 4-9 µm with clamp connections. Kwon *et al.* (2017) also examined ten isolates of *Athelia* spp. isolated from *Ipomoea batatas* showing symptoms of stem rot disease. The microscopic observation showed that the hyphae has a clamp connection, and the speed of mycelial colony growth on PDA media varied from 1.2-1.5 mm/day. In addition, the shape, color and size of the sclerotia also varied between isolates. The sclerotia size was observed macroscopically and the diameter was 0.5-2 mm, while the colors ranged from light brown to dark brown, and the shapes were oval and round.

Molecular identification of SKT isolates.

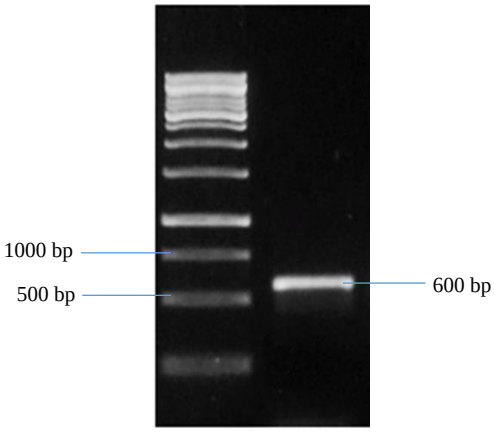

Fig. 4. PCR amplification of 18S rRNA genes with ITS1 and ITS4 primers.

The molecular identification of SKT isolates was carried out based on genetic analysis using primers that amplified internal transcribed spacer (ITS) 18S rRNA genes, namely ITS 1 primer (F: 5'–GGAAGTAAAAGTCGTAACAAGG–3') and ITS 4 (R: 5'–TCCTCCGCTTATTG ATATGC–3') which produced 600 bp DNA fragments (Fig. 4). The DNA fragments produced were purified and sequenced for identification based on similarities to other identified fungal species (Table 1).

Table 1. The 18S rRNA gene sequence for *A. rolfsii*.

The 18S rRNA gene sequence	5'- GAGAAAAGATTATATATGCGAGGAGTTGTGCTGGTAATGAATATTGCATGTGCACACTCTGGAGCT ATATAATATATACACCTGTGAACCAACTGTAGTCAGGAGAAATCCTAACTATGATCACCCTATATA ACTCTTATTGTATGTTACATAGAACGATCTCATATTGAAACTTTGTTTTCTGACAAGTTTCTCTTA ATTAAAAATATACCACTTTCCACCACGGAACTCTTGGCTCTTGCCTCCATTAAAAACGCAACGAAA TGCCATAAGTAATGGGAATTTCAAAATCCCGTGAATCCTCCAATCCTTGAACGCCCCTTGCCCCCT TTGGTATTCCCAGGGGCATGCCTGGTTGAAAATCATTAAATTCTCCACCTTACCAATTTTTGGATT TGGCAAGGGTTGGATGGGAAAATTGCTGGGTAGAGTATATTCTGACTGGCTCTCTTTAAAACTATT AATAAGACATGGAGAAATGCCTACCGGTGGTGTGATAATATGTCTACCCCTATTCCGGAAGGGGAA TCTAGCTTGGATGGACTACTTATAAAATCATGCCCATATATCTAGCATATAAATGCATATATTGAC CATTTGACCTCCAATCAAGGAAGACTACCCGCTGAACTTAAGCATATCCATAACCGGAAGAAA–3'

Table 2. Percentage of skill isolate SKT with some homological DNA sequences in GenBank based on 18s rRNA gene sequences.

No	Isolate	% of similarity	Accession number
1	*Athelia rolfsii* isolate SR2	98	JN241555.1
2	*Athelia rolfsii* isolate BOScR1	98	KJ546416.1
3	*Athelia rolfsii* strain SR1USVL	98	KU128903.1
4	*Athelia rolfsii* isolate AS 1	98	JN241563.1
5	*Athelia rolfsii* isolate 3095	98	JN241556.1
6	*Athelia rolfsii* strain A8 2	98	GU08230.1
7	*Athelia rolfsii* 18S	98	DQ059578.1
8	*Athelia rolfsii* strain ATCC 201126	98	AF499018.1
9	*Athelia rolfsii* isolate 176	98	JN241564.1
10	*Athelia rolfsii* isolate 3083	98	JN241562.1
11	*Athelia rolfsii* isolate SR001	98	HQ420816.1

Based on the sequence alignment results and the homologous sequences available at GenBank using the BlastN program, the SKT isolates belong to the group *A. rolfsii* isolate SR2 (JN241555.1), *A. rolfsii* isolate BOScR1 (KJ546416.1), *A. rolfsii* strain SR1USVL (JN241555.1), *A. rolfsii* isolate BOScR1 (KJ546416.1), *A. rolfsii* strain SR1USVL (JN241555.1), *A. rolfsii* isolate BOScR1 (KJ546416.1), *A. rolfsii* strain SR1USVL (JN241555.1) KU128903.1), *A. rolfsii* isolate AS 1 (JN241563.1), *A. rolfsii* isolate 3095 (JN241556.1), *A. rolfsii* strain A8 2 (GU080230.1), *A. rolfsii* 18S (DQ059578.1), *A. rolfsii* strain ATCC 201126 (AF499018.1), *A. rolfsii* isolate 176 (JN241564.1), *A. rolfsii* isolate 3083 (JN241562.1), *A. rolfsii* isolate SR001 (HQ420816.1) with a maximum identity level of 98% (Table 2).

The phylogeny tree analysis using the Maximum Parsimony (MP) method with 1000 bootstrap replications showed that the SKT isolate was *A. rolfsii*, because it was in one clade with the *A. rolfsii* mushroom sequences in GenBank with 100% bootstrap support as shown in Fig 5. Based on molecular identification, the SKT fungus isolates obtained from legume plants showing symptoms of stem rot is *A. rolfsii*. Meanwhile, Kwon *et al.* (2017) reported that the phylogeny tree analysis showed that *Athelia* sp. and other Basidiomycetes members are divided into three clusters or groups namely S1, S2 and S3 clusters. *A. rolfsii*, *S. rolfsii*, *Sclerotium delphinii* and *Sclerotium coffeicola* are in the same cluster namely S3 which is the biggest cluster with a bootstrap value of 100%, while *Sclerotium denigrans*, *Sclerotium cepivorum*, *Sclerotinia sclerotiorum* and *Sclerotium perniciosum* are grouped into cluster S1 along with *Monilinia fructicola* and *Botryotinia fuckeliana* with 97% bootstrap values.

Furthermore, *Sclerotium hydrophilum*, *Sclerotium rhizodes*, *Thanatephorus cucumeris* and *Ceratorhiza oryzae-sativae* are grouped in cluster S2 with a bootstrap value of 96%.

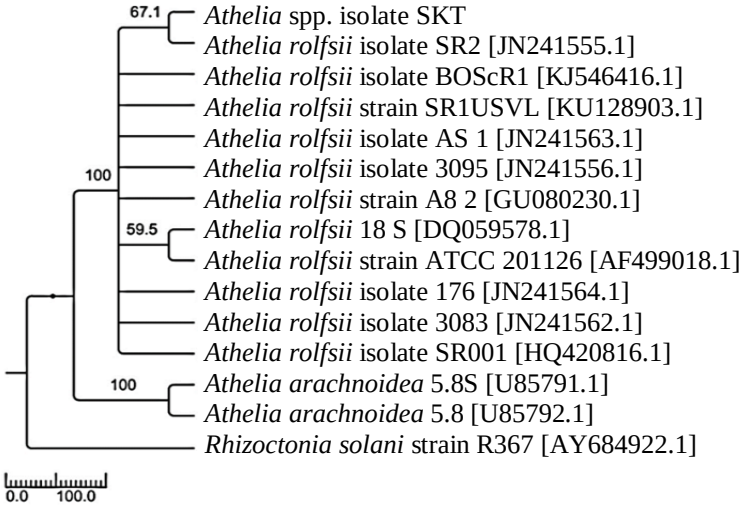

Fig. 5. The phylogeny tree was based on the maximum parsimony (MP) method for 18S rRNA gene sequences which showed a relationship between SKT isolates and fungi in GenBank. Bootstrap value (%) is based on 1000 replications.

Hanafiah *et al.* (2021) on the phylogenetic relationship of class Basidiomycetes and ordo Atheliales including *Athelia arachnoidea* (AF518601), *Athelia decipiens* (AY586632) and *Athelia epiphylla* (AY586633), showed that the DNA sequences were in one clade in the GenBank database with 98% bootstrap support. Furthermore, Nugroho *et al.* (2019) reported that the phylogenetic analysis of *Athelia* spp. ITS sequence with *Sclerotium delphinii* and *Sclerotium coffeicola* form one cluster with 99% bootstrap support. *S. rolfsii* MHGNU F117 from *Cucumis melo* (KU760983) is in a similar clade with fungi sequences (KP257582), *S. rolfsii* KACC47819 from *Momordica charantia*, (HM355751), *S. rolfsii* KACC42087 from *Hosta plantaginea*, (K259782), and *Catharanthus roseus* in the GenBank database with 92% bootstrap support, and also with (AB075316) *Sclerotium delphinii* 196 from *Lycopus* sp. with 83% bootstrap support as well as *Athelia epiphylla* with 60% bootstrap support.

CONCLUSION

The isolate of pathogens causing stem rot disease in Bali's local legume plants produced six fungal isolates coded SKT, SKB1, SKB2, SKB3, SKL and SKN isolates. SKT isolates had the highest virulence rate based on the pathogenicity test of peanut plants. Meanwhile, molecular identification shows that this isolate is *Athelia rolfsii*, because it is in a similar clade with *Athelia rolfsii* fungi sequences in GenBank with 100% bootstrap support.

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