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First Report of Curvularia specifera the Cause of Leaf Spot Disease on Rice in Bali, Indonesia

I Gusti Agung Gede Bawa[1] *Dewa Ngurah Suprapta[2] I Made Dira Swantara[1] I Gede Rai Maya Temaja[2] Khamdan Khalimi[2]
1. Department of Chemistry, Faculty of Mathemathic and Natural Science, Udayana University, Campus Bukit Jimbaran Badung Bali Indonesia
2. Laboratory of Biopesticide Faculty of Agriculture Udayana University, Jl. PB. Sudirman Denpasar Bali Indonesia

Abstract
Leaf spot disease was found to occur on rice grown in Badung Regency, Bali Indonesia at the end of 2016. A fungus was isolated from diseased-rice plant and was confirmed to cause inhibition to rice seed germination. More than 63% of the rice seed did not germinate after inoculated with the isolated fungus. Based on the Koch's postulate test, the fungus caused of leaf spot disease symptom that appeared on the 3rd day with the disease intensity reached 34.26% on the 14th day. This fungus could also cause sheath spot disease on the 14th day. This study aimed to identify the species of fungus causing the leaf spot disease in rice plants. Based on macroscopic, microscopic and analysis of 18S rRNA gene, it was concluded that the fungus causing leaf spot disease in rice plant of Ciherang cultivar in Badung regency, Bali Indonesia is *Curvularia spicifera*.
Keywords: leaf spot disease, rice plant, *Curvularia spicifera,*

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods in the world after wheat which is consumed by more than three billion people worldwide each day. In Indonesia, rice is a strategic commodity, as it is consumed by almost 90% of its community, therefore it has a great influence on economic, politics, and security stabilities (Ismunadji and Partoharjono, 1988). Various important factors can reduce rice production, among others insects, diseases, and weeds (Yamaguchi *et al.,* 2008). Rice diseases are mainly caused by fungi, bacteria and viruses. One of the diseases that is caused by fungi in rice plants is leaf spot disease. This disease can be caused by various types of fungi, among others brown leaf spot disease caused by *Cochliobolus miyabeanus* fungus (Ito and Kuribayashi) Drechs. ex Dastur (Anamorph *Bipolaris oryzae* (Breda de Haan) Shoemaker (Fazlu and Schroeder, 1966; Hossain*et al*., 2011; Groth and Hollier, 2015), *Curvularia lunata* (Tann and Soytong, 2017), *Curvularia oryzae* (Meethongkham and Soytong, 2013), narrow brown leaf spot disease caused by *Cercospora oryzae* fungus (Hossain*et al*., 2011; Faruq*et al*., 2015); and blast leaf spot disease caused by *Pyricularia oryzae* (Pandey, 2015).

The presence of leaf spot disease was very detrimental to farmers, because it greatly affects the quantity and quality of the harvest (Kusain*et al*., 2015). Brown leaf spot disease in rice plants caused by *Bipolaris oryzae* could reduce the crop yields up to 90% in Asia (Mew and Gonzales, 2002), whereas, in Morocco this disease was caused by *Drechslera oryzae* causing yield lost for 75% (Kohls*et al*., 1987). Blast disease caused by *Magnaporthe oryzae* B. Couch, anamorph *Pyriculariaoryzae* Cavara could cause yield lostfor 50-90% (Agrios, 2005; Chaudhary *et al*., 1994), and even reached 100% (Prabhu*et al*., 2009; Fisher *et al*., 2012) in the worldwide rice cultivation area.

Differ to the diseases of brown leaf spot, narrow brown leaf spot, and leaf blast, the disease of leaf spot found in Badung Regency, Bali Indonesia showed the symptom of a long and wide grayish white spots surrounded by brown color with irregular spot shape, as shown in Figure 1.

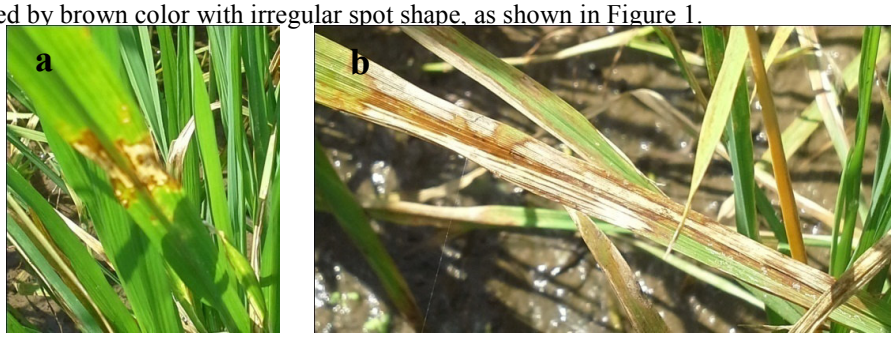

Figure 1
The symptoms of leaf spot diseases found in the field. The spots were grayish white which were surrounded by brown color with irregular shape spot. a: Early stage symptom; b: Severe symptom
(Source: private collection, 2016)

The characteristics of the fungi causing the leaf spot disease found in the field rice were black chromatic colony as the base; on the surface of the media there was a grayish white chromatic mycelia growing with white chromatic margins (Fig. 2).

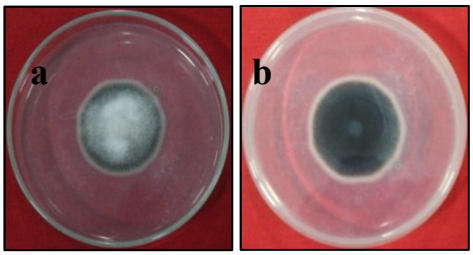

Figure 2
Macroscopic characteristics of isolated fungi: (a) upside view of fungal colony in PDA medium; (b) downside view of the colony

In Koch's Postulate test, the symptoms of leaf spot disease appeared on the 3rd day after inoculation (Figure 3 (a) and on the 14th day, the disease intensity reached 34.26%. In addition, the symptoms of spot disease was appeared on the sheath on the 14thday after inoculation (Figure 3 (b). These fungus could also inhibit the germination of the seeds of rice cultivar Ciherang. The percentage of seeds that did not germinate was 63.89%, and hence, this caused rotting sprouts disease that appeared on the 8th day after inoculation (Figure 3 (c).

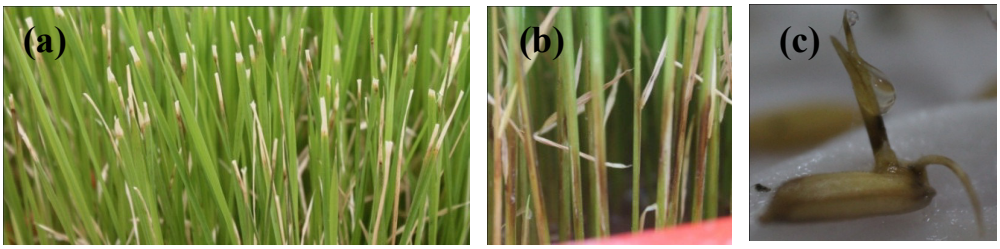

Figure 3
The disease symptoms in Koch's Postulates : (a) leaf spot; (b) sheath spot; (c) rotting sprouts diseases

Based on the numbers of diseases caused by those fungi and the great potency of agricultural production losing, therefore the identification for finding out the name of the fungus species causing the leaf spot disease on rice plants was very important to perform in order to facilitate the control.

2. Research Methods

Isolation of Fungus

The fungus was isolated from the parts of the rice leaves showing leaf spot disease symptom. The infected rice leaves were collected from the rice plants cultivated at Subak Ayunan, Village of Ayunan, Abiansemal District, Regency of Badung, and Province of Bali-Indonesia. The infected leaves were taken and washed with running tap water followed by rinsing with sterile water. The leaves were then dipped into sodium hypochlorite (NaOCl) solution and rinsed three times by sterile water. The sterile leaves were dried and then a small pieces (0.5 x 0.5 cm) were cut in the part of between the healthy and infected leaf. The small pieceswere thenplantedin Potato Dextrose Agar (PDA) medium and incubated at 25±2°C for 7 days. If there was a contaminant, the fungal colonies with the diameter of 5 mm was replanted on new PDA media and then incubated at 25±2°C. This stage was conducted repeatedly until a pure isolate was obtained. This pure isolate was inoculated at a 14-day old healthy paddy seed for confirming the similarity of the symptom in the field. For this purpose,the Koch's postulates test was performed.

Identification of Fungi Causing Leaf Spot Disease on Rice Plants

The macroscopic identification was conducted by observing the color of colonies, colonic inverted color, radier lines or concentric, the surface of the colony, and the growth of fungus. Microscopic identification was done by observing the shape of hyphae or spore under microscope and analysis by Scanning Electron Microscope (SEM) method.

Molecular identification was performed using PCR method with working procedure including: (1) DNA fungal extraction; the isolated fungus was inoculated in a 1.5 mL Eppendorf tube containing 0.5 mL of PDB medium plus chloramphenicol and incubated overnight in a orbital shaker at 150 rpm and 30°C. Then the fungal cultures were arranged photometrically (absorbance at 530 nm, McFarland 0.5 standard) so that the concentrations were ranging from $1x10^6$ to $5x10^6$ cells/mL. In the case of a filamentous fungus, the conidia was separated from the outside of the mycelium by filtering through sterile glass wool. Ten fold dilutions (10^6-10^0

cells) were performed to examine the sensitivity and specificity of the test.The fungal suspension to be determined was centrifuged at 5000×g, Thenpellet was frozen at 220°C for one hour and incubated at 65°C for one hour in 0.5 extraction buffer (50 mMTris-HCl, 50 mM EDTA, 3%sodium dodecyl sulfate, and1% 2-mercaptoethanol).Lysate was extracted with chloroform-isoamilalcohol (25: 24: 1 v/v/v).After that, 65 mL of 3 M sodium acetate and 75 mL of 1 M NaCl were added to 350 mL of supernatant, and then incubated at 4°C for 30 minutes. The DNA was recovered by precipitation with isopropanol and washed with 70% ethanol; (2) PCR analysis; the DNA extract was amplified using Robo Cycler 96 temperature cycles (Stratagene, La Jolla, Calif).The primers used were ITS1 (forward) (5 'TCC GTA GGT GAA CCT GCG G 3'),hybridizing with the final end of 18S rRNA,and ITS4 (reverse) (5 'TCC TCC GCT TAT TGA TAT GC 3') (Life Technologies, Barcelona, Spain)with PCR product of± 500 bp. Fifty milliliters of PCR mixture containing 10 mL of DNA template, 6 mL of 25 mM $MgCl_2$, 5 mL PCR buffer without $MgCl_2$; 200 mM of each deoxynucleoside triphosphate, 25 pmol of each primer, and 1 U Taq DNA polymerase (Biotools B & M Labs, S.A., Madrid, Spain) were analyzed by PCR instrument. The reaction was carried out within one cycle at 95°C for 5 minutes as pre-denaturation stage, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing stage at 55°C for 1 minute, and extension stage at 72°C for 1 minute, followed by 1 cycle at 72°C for 6 minutes. (3) Detection of amplification products; 10 mL of the amplification product liquid was electrophoretically separated by agarose gel 2% containing 13-Trisborate-EDTA buffer and visualized using ethidium bromide under UV light. The molecular weight of each DNA was observed. (4) DNA sequencing for determining the identity of the fungus. Nucleotide was sequenced by ABI-Prism 3100-Avant Genetic Analyzer. DNA sequences were then trimmed and assembled with the application of ChromasPro version 1.5 program.

The data that has been assembled was then BLASTed with the registered data in the NCBI (National Center for Biotechnology Information) through the website http://www.ncbi.nlm.nih.gov/. Some of the sequences homologous were downloaded from the Genbank data in NCBI. Furthermore, the isolate fungal sequence, homologous sequences from Genbank, and one of outgroup sequence were aligned with CrustalW in MEGA 7 program (Kumar *et al*., 2016) and then bootstrapped using the PAUP 4.0 program so that the phylogenetic tree was found.

3. Results and Discussion

The fungus that has been isolated from the rice leaf of cultivar Ciherang showed the characteristic as follows : the colony color was black in the base, on the surface of the medium there was grayish white mycelium with white margins growing (Figures 2a and b). The fungal growth in 3 days reached 29.61 mm following the linear regression equation: $Y = 6.54X + 9.98$ with regression coefficient (R^2) = 0.95; where Y is the diameter of fungi colony (mm) and X is the time of inoculation (day). This fungus had brown hyphae and branched; forming conidia or brown cylindrical spored with 3-septate, in which the center cells were larger than the end cells (Fig. 4a, b, and c).

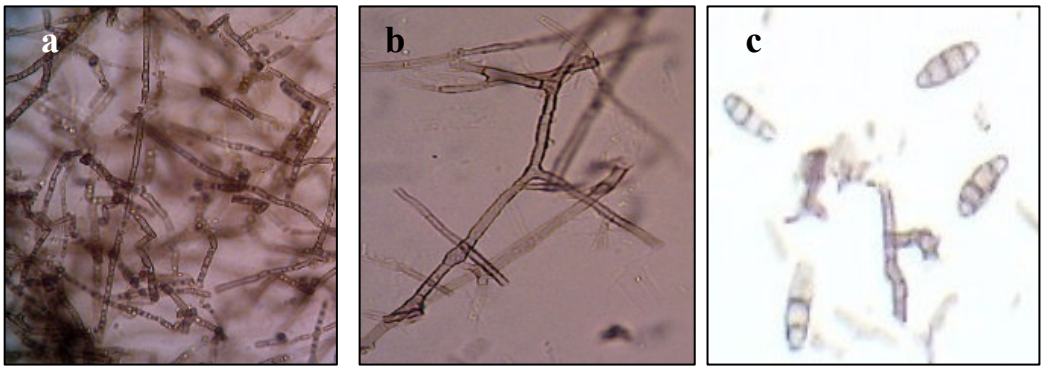

Figure 4
Microscopic features of the isolated fungi: (a) mycelia; (b) hyphae; and (c) septate conidia.

Scanning with the electron microscope (SEM) indicated that this fungi hadbranched hyphae and conidiophore at the tip (yellow arrow), as shown in Figure 5.

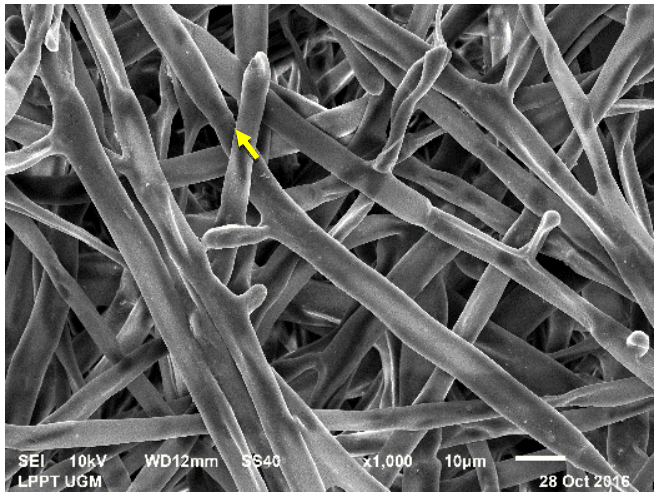

Figure 5
The hyphae dan mycelia of the fungus observed under Scanning Electron Microscope. Arrow indicates the hypae of the fungus.

Molecular identification result indicated that the isolated fungal sequence, hereinafter called *Curvularia* Bali Rice, had proximity to some fungi of the *Curvularia* genus (Table 1).

Table 1
Comparison of the percentage similarity of *Curvularia* Padi Bali sequences with some DNA sequences in GenBank

Fungi Species	Similarity (%)	Accesion Number
Curvulariaverruculossa strain LSF 9	100	KU647724.1
Curvulariaoryzae CBS 169.53	100	NR138221.1
Curvulariaverruculossa isolate CBS 150.63	100	KP400652.1
Curvulariaoryzae isolate CBS 169.53	100	KP400650.1
Curvulariaverruculossa strain CBS 149.63	100	HF934909.1
Curvulariaverruculossa strain FMR 11526	100	KP131994.1
Curvulariaoryzae isolate 2715	100	EU272519.1
Curvulariatsudae ATCC 44764	100	NR147464.1
Curvulariaspicifera isolate Cs-A	100	KX270359.1
Curvularialunata isolate UFMG PNA	100	KY364633.1
Curvularialunata isolate UFMG PEZ1	100	KY364623.1
Curvulariatsudae isolate E-527	100	KU059949.1
Curvulariatsudae isolate E-516	100	KU059944.1
Curvulariaverruculossa strain CBS 150.63	98	HF934908.1

The results of phylogenetic analysis using the MEGA 7 and bootstrap with PAUP 4.0 program indicated that *Curvularia* Padi Bali had proximity to *Curvularia spicifera* with 100% similarity (Figure 6). Based on the phylogeny tree analysis it had been known that the fungus causing the leaf spot disease on rice plants that was found in Subak Ayunan, Bali was *Curvularia spicifera*. This result was reinforced by Jeon*et al*. (2015) who successfully isolated the *C. spicifera* in wheat kernels in Korea, in which the macroscopic and microscopic characteristics of these fungi are almost identical. *Curvularia spicifera* isolated from wheat seeds have morphological characteristics: (1) The color of the colony is dark gray to light, colony diameter on PDA medium is approximately 4-6 mm at 25^{o}C, on 5 days after inoculation. (2) Conidiophore is simple, tense, geniculate, branched, average width of 5.5 µm. (3) Singel conidia at conidiophore tip. (4) Conidia is not curved, oblong to cylindrical and predominantly 3-septate.

The presence of *C. spicifera* in rice plants has not ever been reported yet. This finding is the first report on the presences of *C. specifera* as the cause of the leaf spot disease in rice plant, in particular in Bali, Indonesia. This fungi was reported as the cause of chronic rotten disease in citrus fruits (*Citrus reticulata*) in southern Italy (Garganese*et al*., 2015) and it was also found in wheat kernels in Korea (Jeon*et al*., 2015). However, the presence of diseases in rice plants caused by the *Curvularia* species have been widely reported. Anjana and Anitha, (2012) suggested that *C. lunata*, *C. affinis*, *C. geniculata*, *C. oryzae*, and *C. pallescens*have caused "black kernel" disease in rice plants. Kamaluddeen *et al.* (2013) reported the presence of blight diseases caused by *C. lunata* that can attack the leaves, sheaths and rice grains in Uttar Pradesh, India. In Malaysia, the presence of *C. hawaiiensis*, *C. geniculate*, *C. eragrostidis*, *C. aeria*, and *C. lunata* have caused leaf spot disease in rice

plants (Kusain *et al*., 2015). Tann and Soytong (2017) reported the presence of brown leaf spot disease caused by *C. lunata* has that attack rice plant of IR-66 varieties in Cambodia.

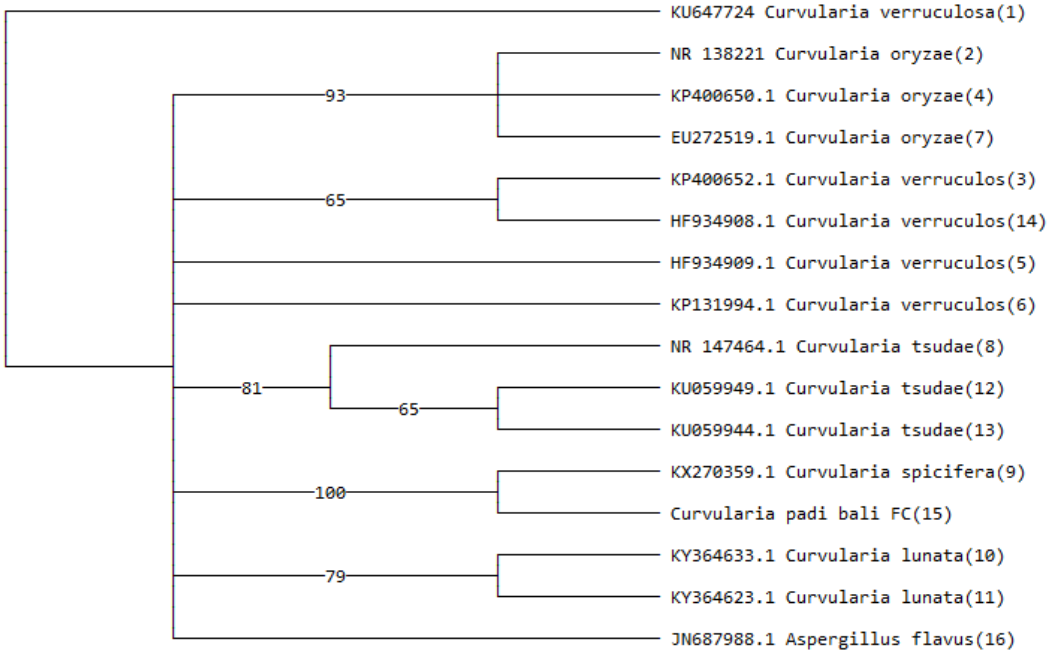

Figure 6
Phylogeny tree of *Curvularia* Padi Bali with maximum parsimony tree method

4. Conclusion

The cause of leaf spot disease in rice plant found in Subak Ayunan, Bali, Indonesia is identified as *Curvularia spicifera*. This is the first report on the presences of *C. spicifera* in the rice plant showing leaf spot symptom. This fungi could cause various diseases in rice plants, such as leaf spot, rotten seed rod, sheath spot disease and could inhibit the germination of the seeds.

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