Rice sheath rot disease etiology and characterization of the pathogen

K Afifah¹*, S Wiyono² and T S Yuliani²

¹Post Graduate student of Phytopathology Study Program, Faculty of Agriculture, IPB University, Bogor 16680, Indonesia
²Department of Plant Protection, Faculty of Agriculture, IPB University, Bogor 16680, Indonesia

*Email: Afifah179@gmail.com

Abstract. Rice sheath rot disease in Indonesia caused by Sarocladium oryzae is currently become a major pathogen. The objectives of this research was to describe disease etiology and characterization of rice sheath rot pathogen. Characterization was conducted by isolation followed by morphology-based identification, and observation of rice sheath rot symptoms. The fungal colonies has 5 different morphotypes, namely KP, KP2, KP3, PW03, and PW3 which all were pathogenic to rice. Morphotypes of KP3 and PW3 colonies had yellowish color; while KP, KP2 and PW03 colonies had orange color. Microscopically conidia of KP, PW3, and PW03 had longer and thinner form; while KP2 and KP3 were shorter and wider. PCR confirmation using specific primer for S. oryzae resulted that all tested isolates were S. oryzae. The disease occurred on uppermost leaf sheath enclosing the young panicles. The lesions appeared as oblong with brown margins and grey centres. The panicles infected by S. oryzae were chaffy, discoloured, and shrivelled. The incubation period of this disease was 3 to 4 d.

1. Introduction

Previously, rice sheath rot caused by Sarocladium oryzae was considered as the major disease in Indonesia. Lately, infection of S. oryzae was reported as the major constraint in rice production in some rice-producing countries. Rice cultivation infected by S. oryzae may cause yield loss up to 85% [1]. Moreover, infection of S. oryzae affects empty panicle and reduction in seed weight [2], thus causing abnormal rice germination [3]. Rice sheath rot disease was first reported in Indonesia in 1987 by Indonesia-Japan study collaboration in food crop protection (ATA-162) during dry season with moderate incidence status [4]. Climate change, cropping patterns, and introduction of new cultivars are among several causes of the increasing of this disease. Rice sheath rot disease could now be easily found in Indonesia and its impact to yield loss is important to be observed [5]. The more observation on disease etiology and the causal pathogen, the sooner the controlling strategies can be obtained. The etiology includes recognition of symptoms caused by pathogen, pathogen incubation period, and pathogen transmission. Pathogen characterization comprises color and edge of the colony, mycelium growth, the shape and size of conidia which includes colony diameter. The objective of this research was to describe etiology of rice sheath rot disease and characterize S. oryzae.
2. Methods

2.1. Fungus isolation
Rice samples were collected from rice cultivation in Karawang and Cianjur Regencies. Grain and sheath with rotten symptom were isolated by washing with water to remove dirt, followed by surface sterilization with 2% NaOCl for 2 min, and rinsed twice with sterile water then dried in room temperature for 10 min. The sterilized samples then cut in size of 1 cm² (sheath) and no cut (grains), then plated on potato dextrose agar (PDA) media for 3 to 4 d. Colonies with similar characteristic with S. oryzae then recultured in a new PDA [6].

2.2. Visual inspection of sheath rot symptoms
The objective of this inspection was to ascertain the symptoms of rice sheath rot are caused by S. oryzae. One sample rice sheath with rotten symptom was cut into 2 pieces in size 0.3 cm² for each piece. The first piece was placed on glass object that has been dripped with sterile water and covered with a cover glass, then observed under a compound microscope. If rice sheath is infected by bacteria, the sheath edge will release ooze. While, if it is infected by fungi, there is mycelia or conidia observed. The second piece was cultured on PDA media to ensure the microbes that colonize the rice sheath [7].

2.3. Pathogenicity test
Pathogenicity test was carried out by inoculating S. oryzae isolates to 80-days-old rice (var. IR 64). Beforehand, the rice sheath was surface sterilized by spraying 70% alcohol, then rinsed with sterile water. The 14-days-old S. oryzae isolates were inoculated to the inoculation area then wrapped with wetted tissue paper. The inoculation area was then uncovered from the tissue paper after 24 hr. The symptom occurrence was observed every day until 14 d after inoculation [8]. The symptom for pathogenic isolates is brown spots formed on the rice sheath.

2.4. Fungus identification
Microscopic identification was carried out using transparent tape technique, while macroscopic identification was done by observing the color, diameter, and shape of the colony on PDA 14 d culture. Transparent tape technique was firstly conducted by dripping water and lactophenol cotton blue on the glass object. Subsequently, transparent tape was attached slowly to the culture then pinned to glass object. Observations were conducted using compound microscope by measuring 20 conidia for each [9].

Molecular identification was conducted by extracting the fungus DNA using Qiaamp DNA mini kit (Qiagen). DNA amplification was carried out on Sensoquest Thermal Cycler PCR machine that followed the Q5 protocol from New England Biolabs (NEB). Amplification was done using specific primer for S. oryzae, i.e. forward primer (5’- GAT CTC TTG GCT CTG GCA TC -3’) [10] and reverse primer (5’- GCT CCT GAG GGT TGA AAT GA - 3’) [11] with 157 bp amplification target. DNA amplification reaction was carried out with a total volume of 25 μL consisting of 12.5 μL Q5 hot start high-fidelity 2X master mix, 1.25 μL forward primer, 1.25 μL reverse primer, 2 μL DNA samples, and 8 μL nuclease free water. DNA amplification used PCR program by preheating to reach a temperature of 98 °C takes 30 sec, followed by 35 amplification cycles in which each cycle consists of denaturation of 98 °C for 10 sec, then followed by annealing of 60 °C for 20 sec, and extension at 72 °C for 25 sec. The final extension was performed at 72 °C for 2 min and at the end of the cycle the temperature was maintained at 4 °C to 10 °C.

The amplified product was analyzed on 2% agarose gel (0.4 g agarose and 20 mL TAE buffer 1X). Electrophoresis was conducted at 94 volts for 40 min and the agarose gel was then incubated in a dye containing 1% ethidium bromide for 40 min, and then washed with water. The results were visualized with an ultraviolet transilluminator. The target DNA bands on electrophoresis were documented with a digital camera.
2.5. Data analysis
Data was analyzed descriptively with statistical tools i.e., frequency and average using Microsoft Excel 2013.

3. Results and discussion

3.1. Causes of rice sheath rot
Rice sheath rot symptoms caused by *S. oryzae* were indicated by brown spots on the sheath with gray centers and white mycelium, panicle discoloured, and if there is a severe infection in rice cultivation, only half of panicles will be emerged. Grain and rice sheath rot were the effect of helvolic acid and cerulenin activities produced by the pathogens [6].

![Figure 1. Rice sheath rot symptoms on: (a) sheath, (b) panicle, (c) severe infection.](image)

Based on observation of 20 symptomatic sheaths it was found that 62.5% of rice sheath rot symptoms from Karawang and Cianjur Regencies were caused by *S. oryzae*. This data was supported by the appearance of oval-shaped, aseptate, hyaline conidia found on the edge of sheath pieces. However, *Fusarium* sp. were also detected in this initial observation (Table 1).

| Observation method | Percentage of infected sheath by the fungi<sup>a</sup> |  |
|--------------------|---------------------------------|---|
|                    | *S. oryzae* | *S. oryzae* and *Fusarium* sp. | *Fusarium* sp. |
| Microscopic        | 65          | 20                         | 15               |
| Macroscopic        | 60          | 30                         | 10               |
| Average            | 62.5        | 25                         | 12.5             |

<sup>a</sup>Samples were 20 symptomatic rice sheaths from Karawang and Cianjur Regencies.

3.2. Morphotype of *S. oryzae*
Fungi *S. oryzae* generally develops orange colony color with slow mycelium growth [11]. However, the obtained fungus colony from isolation of symptomatic sheaths and grains (KP, KP2, KP3, PW03 and PW3) showed varying colors and mycelium growth (Fig. 2). The front side of all colonies was white, while the back side of morphotypes KP3 and PW3 colonies have yellowish color with faster mycelium growth; and the other KP, KP2, and PW03 colonies have orange color with slower mycelium growth.
The obtained fungus morphotypes have different length and width of conidia. Morphotypes of KP2 and KP3 have wider and shorter form of conidia compared to KP, PW3 and PW03 which have tighter and thinner form (Fig. 3).

The cell structure of *S. oryzae* consisted of hyaline conidia, aseptate and cylindrical, conidiophores arise from hyphae, hyphae are septate and conidia arise from conidiogen cells (Fig. 4).
Table 2. Morphotypes of fungus *S. oryzae* from Karawang and Cianjur.

| Morphotype | *S. oryzae* colony | Size of conidia |
|------------|--------------------|-----------------|
|            | Back side of colony | Edge type of colony | Diameter (cm) | Width (µm) | Length (µm) |
| KP         | Orange-white       | Regular          | 3.90          | 0.65       | 3.93        |
| KP2        | Orange with brownish spots | Regular | 3.50          | 0.76       | 2.64        |
| KP3        | Bright yellowish white | Irregular | 5.15          | 0.86       | 2.48        |
| PW03       | Brownish orange shaped like a flower | Irregular | 3.65          | 0.81       | 3.87        |
| PW3        | White and yellow   | Regular          | 6.00          | 0.66       | 3.25        |

The pathogenicity test of 5 colonies indicated that all morphotypes are pathogenic to rice. Sheath rot symptoms appeared 3 to 4 d after inoculation (incubation period). The difference in grain rot of those morphotypes was estimated due to differences in virulence of each morphotype (Table 3).

Table 3. Pathogenicity of *S. oryzae* isolates in rice cultivation.

| Morphotype | Observed symptoms<sup>a</sup> |
|------------|-----------------------------|
|            | Sheath rot                  | Rice grain rot |
| KP         | +                           | -              |
| KP2        | +                           | -              |
| KP3        | +                           | -              |
| PW3        | +                           | +              |
| PW03       | +                           | +              |

<sup>a</sup>(+) indicates that pathogen can cause symptoms; (-) pathogen cannot cause symptoms.

Target DNA of *S. oryzae*, was successfully amplified from the morphotypes KP, KP2, KP3, PW03, and PW3, i.e. showed by 157 bp DNA bands on agarose gel (Fig. 5).

![Figure 5. Visualization of DNA amplification using specific primer for *S. oryzae*: M DNA marker (100 bp); K1 and K2, positive controls; KP, KP2, KP3, PW03, and PW3 were isolates morphotype.](image)
4. Conclusion
The infected rice sheath by *S. oryzae* were indicated by brown spots with gray centers and white mycelium. The penicles infected by *S. oryzae* are chaffy, discoloured, and shriveled. The incubation period of pathogen *S. oryzae* is 3 to 4 d. The pathogen infecting rice in Karawang and Cianjur can be differentiated into several morphotypes.

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