

First Report of Leaf Spot Disease on *Rhizophora mucronata* from Mangunharjo Village, Semarang City, Central Java, Indonesia

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Abstract. Mangrove diseases are the most neglected threats that might occur in Indonesia's mangrove ecosystems. During a field study at Mangunharjo village, Semarang City, Central Java, Indonesia, some pests and diseases were found in *Rhizophora mucronata*, including leaf spot diseases commonly caused by fungi. This study aimed to isolate, determine, and identify the causative agent of leaf spot diseases. Isolation of the causative agent was done using the surface sterilization method, then all isolates were tested for pathogenicity using detached leaf assay on healthy leaves. Furthermore, morphological and molecular identification based on ITS rRNA gene sequence was done to confirm the species of the causative agent. Three endosymbiont fungal strains were isolated from *R. mucronata*'s leaves with leaf spot disease symptoms. The pathogenicity test confirmed that strain RM1 was the causative agent. The ITS rRNA gene sequence data of strain RM1 had 99.98% similarity to *Pestalotiopsis rhizophorae*.

1 Introduction

Indonesia is home to the world's largest mangrove ecosystem, accounting for 21% of global mangrove coverage, as reported by the Global Mangrove Alliance [1]. These ecosystems play a vital role in protecting coastlines from flooding and erosion, supporting biodiversity, cycling nutrients, and serving as spawning and nursery grounds for numerous organisms. Despite these benefits, mangrove ecosystems face significant threats, including human activities, natural disasters, and disease [2].

Among these threats, mangrove diseases remain one of the most overlooked problems. Common diseases affecting mangroves include leaf spots, leaf drops, necrosis, dieback gall, and cankers, many of which are caused by fungal infections targeting leaves, stems, and roots [3]. In Southeast Asia, fungal pathogens such as *Curvularia* sp., *Colletotrichum* sp., *Neopestalotiopsis* sp., and *Pestalotiopsis* sp. have been linked to mangrove diseases in

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Southeast Asia countries [4], [5]. However, reports of mangrove diseases in Indonesia are scarce.

In a field study conducted in the mangrove ecosystem of Mangunharjo Village, Semarang City, Central Java, Indonesia, leaf spot disease was identified as one of the most significant issues, particularly affecting *Rhizophora mucronata* [6]. This study aims to isolate and identify the fungal endosymbionts associated with leaf spot disease in *R. mucronata*, determine the causative agent, and characterize it based on morphological features and ITS rRNA gene sequencing.

2 Material and methods

2.1 Material

The leaves of *R. mucronata* with leaf spot disease symptoms were collected from the location that has been listed in our previous report [6]. The symptomatic leaves were indicated by the presence of decoloration with circular, semi-circular, or feathery edge shape [5]. Furthermore, the leaves were kept in sterile Ziplock plastic and then transferred to the laboratory. In the laboratory, disease manifestation in the leaves was observed with a stereo microscope. This step was done to differentiate between diseases and pest infestation.

2.2 Fungal endosymbiont isolation and purification

Isolation of fungal endosymbiont from the symptomatic *R. mucronata*'s leaves was done according to Shu *et al.* [7] with some modifications. The symptomatic leaves were cleaned using flowing sterile water to eliminate dirt and other contaminants, then resized to 2×2 cm² using a sterile blade. Then, the samples were treated using the surface sterilization method using 70% alcohol for 30-60 secs, 5% sodium hypochlorite for 15-30 secs, and sterile water for 1 min. Afterward, the samples were placed on a sterilized tissue for drying. The fairly dried samples were placed on Malt Extract Agar (MEA, HiMedia) + 2% Chloramphenicol for 1 min as a control for the surface sterilization step, then transferred onto a new MEA agar for isolation. During the isolation, one MEA agar plate was opened and placed in the laminar airflow as an environmental contamination check. Furthermore, all MEA agar plates were incubated at 30 °C for several weeks. Each mycelium growing around the samples was transferred and purified on MEA plates. Fungal strains from these plates were then compared with those from control plates. Only fungal strain exhibiting distinct morphological features differing from those on the control plates were further subcultured on fresh MEA plates to obtain pure colonies.

2.3 Pathogenicity test

A pathogenicity test was conducted using a modified detached leaf assay with a mycelial disc according to Liu *et al.* [8]. Freshly picked and healthy leaves of *R. mucronata* were cleaned using sterile water and 70% ethanol. The 7-day-old fungal strains were cut using a cork borer with a diameter of 5 mm and then placed on a leaf. Each leaf was inoculated with four mycelial discs with two replications. The inoculated leaves were kept inside a sterile plastic container with a moistened tissue and then incubated at 27 °C for 5 days. The symptomatic area on the leaf was calculated by measuring the diameter of the necrosis or decoloration surrounding the mycelial disc.

2.4 Identification of causative agent

A fungal identification was carried out on the strain that tested positive in the pathogenicity assessment. Molecular identification followed our established protocols [9]. Fungal DNA was extracted using Chelex 100 and then amplified with a PCR Thermal Cycler utilizing ITS1 (forward) and ITS4 (reverse) primers. The resulting PCR product was sent to 1st Base Laboratory Sdn Bhd in Malaysia for sequencing. Following this, the ITS rRNA gene sequence was aligned using the MEGA X software package. A Basic Local Alignment Search Tool (BLAST) from the NCBI Gene Bank was employed to identify homologous species. A phylogenetic tree was constructed with the MEGA X software, utilizing a neighbor-joining algorithm with a bootstrap value of 1000 repetitions. Additionally, morphological observations were conducted to examine the conidia.

3 Result and discussion

3.1 *In situ* documentation of leaf disease on *R. mucronata*

While less frequently documented than in agricultural systems, leaf spot disease has been consistently observed in mangrove ecosystems. Leaf spot disease refers to various microbial infections that manifest as discoloration lesions or spots on foliage. These infections can induce a range of color changes, including chlorosis that presents as yellow or pale yellow, and necrotic lesions appearing reddish, grey to greyish, brown to dark brown, or even black. The lesion margins are often irregular, reflecting the complex interactions between the host plant and the pathogen involved [10]. Notably, the first recorded instance of mangrove diseases occurred in *Rhizophora mangle* from Puerto Rico in 1920 [11]. It is worth mentioning that fungal infection causes brown, black, rufous, or red spot diseases on the mangrove's leaves [5], [12]. During a field study in a mangrove forest in Mangunharjo Village, some *R. mucronata* trees were allegedly infected by a leaf spot disease (Fig. 1).



Fig. 1. In situ documentation of the leaf spot diseases in *R. mucronata* (Yellow arrows show the targeted leaf spot diseases)

Figure 1 shows the morphology of the infected leaves, which exhibit round to oval spots characterized by rufous pigmentation, irregular margins, and a slightly sunken adaxial surface. These are the common symptoms related to leaf spot diseases, which have also been documented in mangrove [5], [13].

3.2 Pathogenicity test

Fungal endosymbionts from the infected leaves collected in Mangunharjo village were inoculated to ascertain the causative agent. Fungal endosymbionts are defined as fungi that live within the body or cells of their host and form a symbiotic relationship. In this study, fungal endosymbionts were isolated using the surface sterilization method to ensure that the fungi were from the inside of diseased leaves. Three fungal strains were successfully isolated and then applied for a pathogenicity test. The result of this test is shown in Fig. 2.

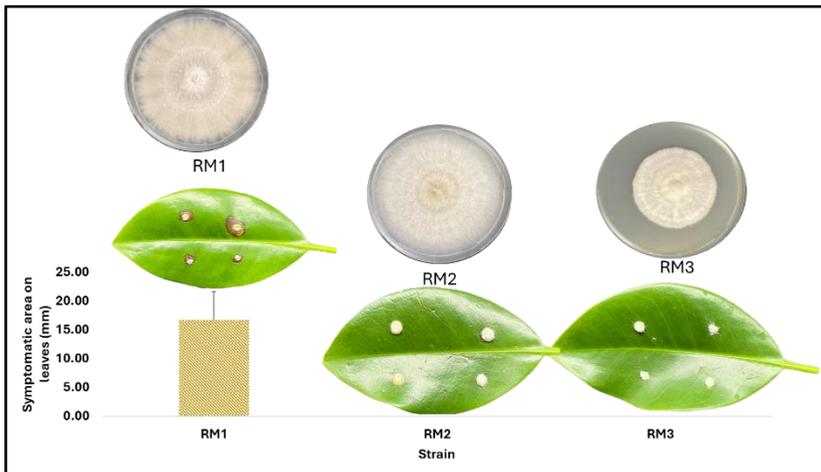


Fig. 2. Fungal strain morphology and the result of pathogenicity test of three fungal endosymbiont strains from symptomatic *R. mucronata*'s leaves

The pathogenicity test successfully demonstrated that among the three fungal endosymbionts isolated from the symptomatic leaves, only strain RM1 exhibited pathogenic potential. Following reinfection, the tested leaves developed symptoms akin to those of the original host, characterized by rufous decoloration with irregular margins, and the spots being slightly sunken. A quantitative study revealed that strain RM1 had an average symptomatic area of $16.75 \pm 4.97 \text{ mm}^2$. The detached leaf assay employing a mycelial disc was chosen to determine the causative agent due to its ease of performance and to give a reliable result. This method is commonly used to investigate disease-causative agents in mangroves and other plants [14]. Furthermore, identification was merely carried out for strain RM1.

3.3 Identification of causative agent

The result of BLAST homology analysis indicated that strain RM1 shares 99.98% similarity with *Pestalotiopsis rhizophorae* MFLUCC 17-0416 and *P. rhizophorae* MFLUCC 17-0417. The reconstructed phylogenetic tree using a neighbour-joining algorithm indicated a stable clade was formed between strain RM1, *P. rhizophorae* MFLUCC 17-0416, and *P. rhizophorae* MFLUCC 17-0417 with bootstrap value ($> 80\%$) (Fig. 3A). Furthermore, the conidia of strain RM1 exhibited pigmentation in the median cells and were characterized by the presence of four euseptate cells (Fig. 3B), which align well with the established characteristics of conidia belonging to the genus *Pestalotiopsis* [15]. The conidia of *Pestalotiopsis* are easily identified using a microscope due to the distinctive morphology, including the presence of four euseptates, pigmented in median cells, and tubular appendages on the apical cells [5], [7], [14], [15].

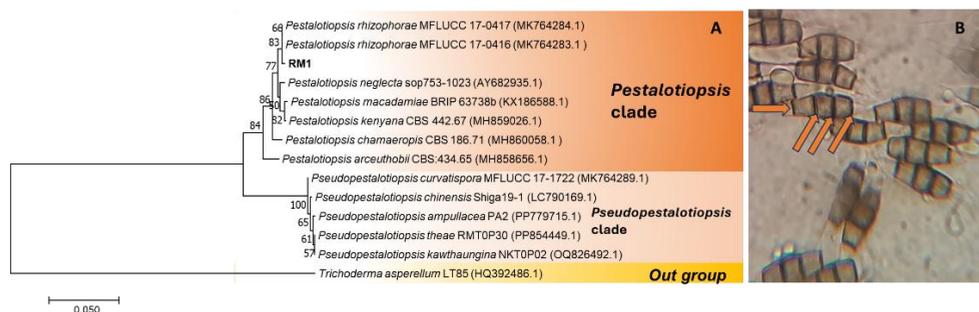


Fig. 3. (A) Neighbour-joining phylogenetic tree of strain RM1 based on ITS rRNA gene sequence with 1000 bootstrap value, (B) conidia morphology of strain RM1 with an optical zoom 40×10 (Orange arrows point the four euseptates)

The infection caused by fungal pathogens such as *Pestalotiopsis*, *Neopestalotiopsis*, and *Pseudopestalotiopsis* often leads to leaf spot diseases [5], [14]. It is highlighted that *Pestalotiopsis* is a common causative agent of leaf spot diseases in various plant species. Previously, Nguyen *et al.* [5] reported *Pestalotiopsis* spp. as fungi associated with certain mangrove species that exhibited leaf spot diseases such as pink and black leaf spots. Also, *P. rhizophorae* MFLUCC 17-0416 and *P. rhizophorae* MFLUCC 17-0417 were first isolated as a leaf spot disease causative agent in *R. apiculata* [13]. The symptoms caused by this fungus were also similar to strain RM1. Therefore, it is suggested that *P. rhizophorae* strain RM1 was the causative agent of leaf spot diseases on *R. mucronata* in Mangunharjo village.

4 Conclusion

This study successfully identified *Pestalotiopsis rhizophorae* strain RM1 as the causative agent of leaf spot disease in *Rhizophora mucronata* from Mangunharjo Village, Central Java, Indonesia. The combination of morphological analysis, ITS rRNA gene sequencing, and pathogenicity testing confirmed its role in inducing characteristic symptoms of leaf spot disease. Future research should focus on understanding the spread and ecological impact of such pathogens and developing effective management strategies to safeguard mangrove ecosystems

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