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DISEASE NOTE

First report of *Lasiodiplodia theobromae* as a causal agent of white-leaf spot on Bitter Ginger (*Zingiber aromaticum*)

Laporan pertama *Lasiodiplodia theobromae* sebagai agens penyebab bercak daun putih pada tanaman lempuyang wangi (*Zingiber aromaticum*)

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ABSTRACT

White-leaf spot symptoms were observed on bitter ginger (*Zingiber aromaticu*), a herbal plant traditionally used in Indonesia. Infected leaves developed white spots with central black dots that progressed to necrotic lesions and leaf perforation. The fungus formed pycnidia on leaves and culture medium, producing oval, brown, septate conidia. Morphological traits and ITS-based phylogenetic analysis identified the pathogen as *Lasiodiplodia theobromae*. The disease, designated as white-leaf spot of bitter ginger, showed optimal fungal growth at 27 °C. This is the first report of *L. theobromae* associated with bitter ginger, highlighting a potential threat to the cultivation and utilization of this important medicinal plant in Indonesia.

Keywords: disease in Zingiberaceae, fungal plant pathogens, morphological and genetic analysis

ABSTRAK

Gejala bercak putih diamati pada tanaman lempuyang (*Zingiber aromaticum*), salah satu tanaman herbal yang banyak dimanfaatkan di Indonesia. Daun yang terinfeksi menunjukkan bercak putih dengan titik hitam di bagian tengah yang berkembang menjadi lesi nekrotik dan akhirnya menyebabkan perforasi daun. Cendawan menghasilkan piknidium pada daun maupun medium kultur, berisi konidium berwarna cokelat, berbentuk oval, dan bersekat. Karakter morfologi bersama analisis filogenetik berbasis ITS mengidentifikasi patogen sebagai *Lasiodiplodia theobromae*. Penyakit ini ditetapkan sebagai bercak putih pada lempuyang, dengan pertumbuhan optimal cendawan pada suhu 27 °C. Laporan ini merupakan catatan pertama asosiasi *L. theobromae* dengan lempuyang wangi, yang berpotensi mengancam budi daya dan pemanfaatan tanaman herbal penting ini di Indonesia.

Kata kunci: analisis morfologi dan genetika, cendawan patogen tanaman, penyakit pada Zingiberaceae

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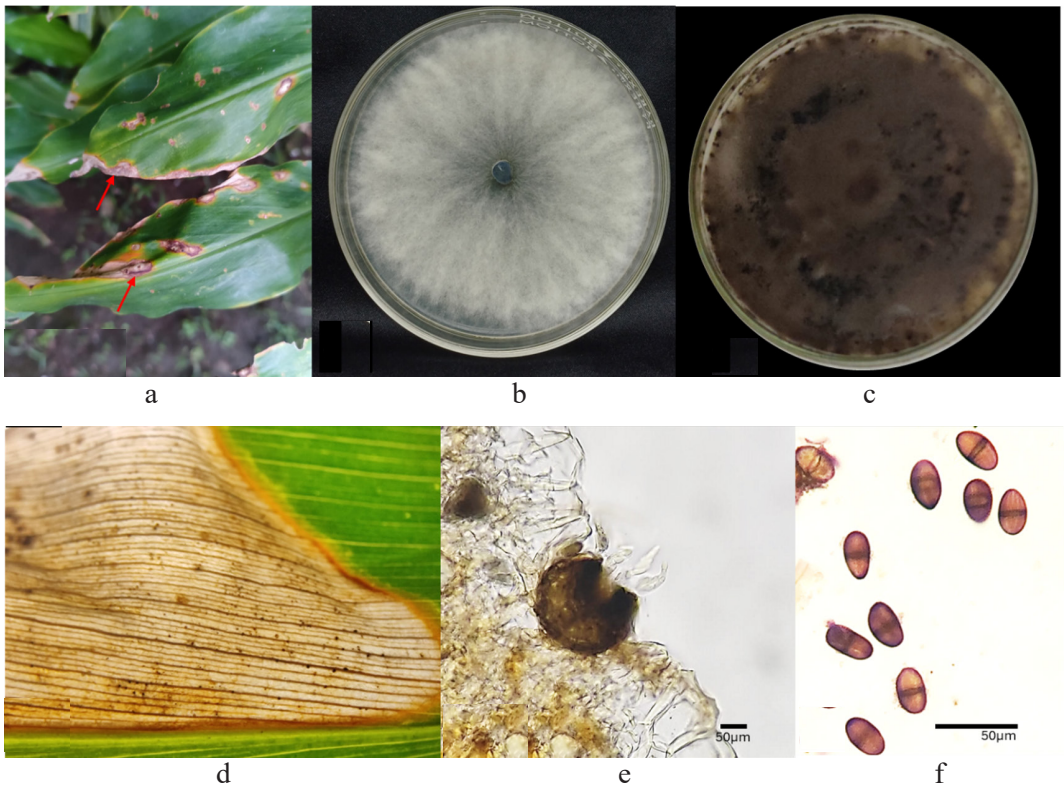
Bitter ginger (*Zingiber aromaticum*), locally known as "lempuyang wangi," is an annual shrubby plant cultivated in Indonesia for its rhizome, which is widely used in traditional medicine to treat various ailments. These species widely distributed throughout the tropics and subtropics, and are especially abundant in Southeast Asia (Sastroamidjoyo 2001; Reddy *et al*. 2024). The rhizome contains essential oils, saponins, flavonoids, and tannins, with zerumbone as the major essential oil component exhibiting anticancer and antitumor activities (Abdul *et al.* 2009; Chandra *et al.* 2003).

Bitter ginger often shows the white leaf spot symptom. In ginger, specifically in *Zingiber officinale*, there are several pathogens that are reported as causal agents of leaf spot disease (Semangun 1992; Wahyuno *et al.* 2020; Hu *et al.* 2023, Yu *et al.* 2023, Wahyuno *et al.* 2023) but there are no reports yet on bitter ginger. Symptoms that we found in the field include white spots on the lamina with central black dots (Figure 1a), progressing to necrotic lesions, and leaf perforation in advanced stages. More than 50% of the plants we observed displayed the symptom, and the severity of the disease ranged from 30% to 50%. In instances of severe disease, the leaves had numerous holes caused by leaf spot, which obstructed optimal photosynthesis. Symptomatic leaves of bitter ginger (five leaves each from five different plants in the same location) were collected in Bogor, Indonesia. The leaf samples were sent to the National Research and Innovation Agency of Indonesia for morphological confirmation of plant identity. The diseased leaf samples were exposed to light for three days to induce conidial formation on their surfaces. Conidia were collected using an inoculation needle and suspended in sterile water in a test tube. The conidial suspension was used for single-conidium isolation. A sterile inoculation needle was dipped in the conidial suspension, then streaked onto a water agar medium, followed by incubation at 25 ℃ for 12 hours. Germinated conidia were then transferred



a b c

d e f

Figure 1 Symptom and morphological characteristics of *Lasiodiplodia theobromae*. a, White-leaf spot symptoms; b, Colony of mycelium on PDA (7-day-old culture); c, Colony of mycelium on PDA (20-day-old culture); D, Pycnidia on symptomatic leaves; E, Pycnidium; and F, Conidia. Scale bar = 50 µm.

to a potato dextrose agar (PDA) medium (Chomnunti *et al.* 2014). Fungal isolation from symptomatic tissues on the PDA medium yielded a grayish-white mycelial fungus (LP1 isolate) (Figures 1c). Optimal growth occurred at 27–28 °C, reaching a colony diameter of 90 mm in 3 days, with inhibited growth outside this range. At first the colony color was white (Figure 1b), then it turned to dark grey overtime (Figure 1c).The fungus produced pycnidia on both infected leaves and PDA medium (Figures 1d, 1e). These pycnidia contained conidia that were initially aseptate and hyaline when young, but became oval, brown, and septate at maturity (Figure 1f). On PDA medium, conidia were formed after 20 days of incubation under a 12-hour light photoperiod, following the darkening of the colony and the development of pycnidia on the medium. The culture has been deposited in IPB Culture Collection, Indonesia.

Koch's postulates were successfully fulfilled. Inoculation of bitter ginger plants was performed using two methods: *in-vivo* and *in-planta*. In the *in-vivo* method, a 10^5 mL suspension of conidia was sprayed onto the entire plant, which was then covered with plastic for 24 hours. The *in-planta* method used the detached leaf technique, where leaves were collected, inoculated with the pathogen colony in the laboratory, and incubated. Inoculation on bitter ginger reproduced symptoms identical to those observed during initial isolation. Symptoms first appeared 10 days after inoculation, but in the detached leaf assay, they developed and spread within 2 days (Figure 2). Reisolation from symptomatic plants yielded colonies matching the original inoculum. Macroscopic and microscopic analyses were performed to identify the fungal isolate as *Lasiodiplodia* sp. Macroscopic observations included colony morphology (color, texture, and growth pattern on PDA), while microscopic analysis focused on spore and hyphal characteristics. This combined morphological approach is a standard method for preliminary identification of plant-associated fungi and has been effectively applied in similar studies (Salvamani and Nawawi 2014).

DNA from the LP1 isolate, cultured on PDA for 7 days, was extracted following the Doyle protocol (1991). PCR amplification targeted the ITS1-5.8S-ITS2 region using ITS4 and ITS5 primers. The use of these primers has been proven effective for identifying plant pathogens, including *Lasiodiplodia* spp. (He *et al.* 2024). The reaction contained 10 µL of

Figure 2 Pathogenicity tests of *Lasiodiplodia theobromae* on bitter ginger using two inoculation techniques. a, Detached leaf assay, control; b, Detached leaf assay, inoculated leaf showing white-leaf spot symptoms; c, *In vivo* assay, control plant (sprayed with sterile water); d. *In vivo* assay, inoculated plant showing white-leaf spot symptoms.

2× MyTaq HS Red Mix (Bioline, Germany), 0.4 µM forward and reverse primers, 1 µL DNA template, and nuclease-free water to a final volume of 20 µL. Amplification was performed in a Labcycler (SensQuest) with the following program: 94 ℃ for 4 minutes; 30 cycles of 94 ℃ for 30 seconds, 55 ℃ for 30 seconds, 72 ℃ for 1 minute; and a final extension at 72 ℃ for 4 minutes (Fujita *et al.* 2001). PCR products were resolved on a 1.5% agarose gel stained with RedSafe™ (Intron Biotechnology), electrophoresed at 50 Volt for 50 minutes, and visualized using a Geldoc Fire Reader V4 (Uvitec Cambridge). Sanger sequencing was performed at a commercial laboratory, and the resulting sequence was deposited in GenBank. The LP1 isolate was identified as *Lasiodiplodia theobromae* via BLAST analysis (GenBank accession LC855210), showing 100% similarity to a Brazilian isolate from *Citrus latifolia* (GenBank ID: MH277691), and further confirmed by phylogenetic analysis (Figure 3). This study is the first to identify *L. theobromae* (Syn. *Botryodiplodia theobromae* Pat. *Diplodia theobromae* (Pat) W. Nowell) as the causal agent of leaf spot disease in the Zingiberaceae family in Indonesia, specifically in bitter ginger (*Zingiber aromaticum*).

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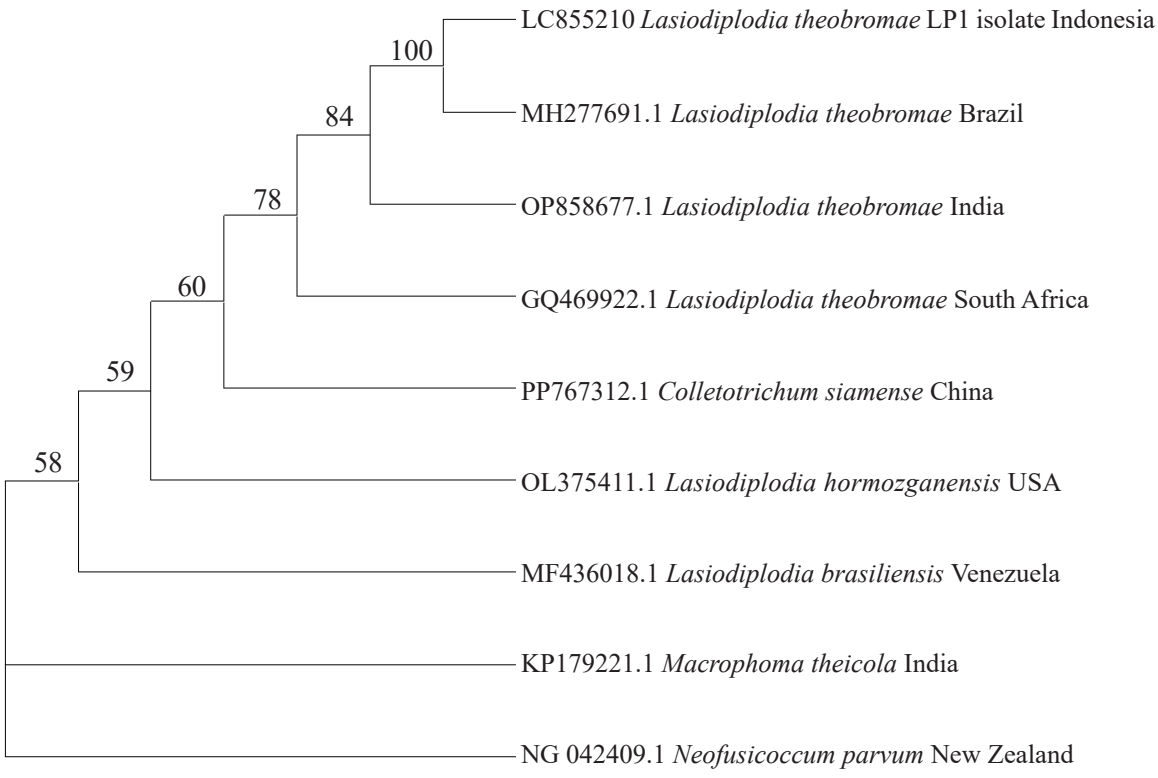

Figure 3 Phylogenetic tree of *Lasiodiplodia theobromae* (LP1) from bitter ginger in Bogor, Indonesia, based on Neighbor Joining analysis using ITS sequences. *Neofusicoccum parvum* isolates were used as the outgroup. Bootstrap values greater than 55% are indicated at the nodes

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