AGROMIX, Volume 16 Number 1 (2025), Pages 1–12

AGROMIX
pISSN (Print): 2085-241X; eISSN (Online): 2599-3003
Website: https://jurnal.yudharta.ac.id/v2/index.php/agromix

Detection and molecular identification of citrus stem rot disease in Bali Province

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Original article

Article history
Received : July 13, 2023
Accepted : March 28, 2024
Published : March 30, 2025

Keyword
Basal stem rot of citrus;
Molecular identification;
Lasiodiplodio theobromae;

ABSTRACT

Introduction: Stem rot disease (SRD) is the primary constraint and limiting factor in national citrus production. This disease is caused by the fungi Phytophthora spp., Diplodia sp., and Botryodiplodia theobromae or Lasiodiplodia theobromae. A study in the Bangli district identified the pathogenic fungi Lasiodiplodia theobromae as the cause of SRD. This research aimed to provide information on the main pathogens and characterization of SRD disease, especially on citrus in Bali. The results of this research can be used as early warnings to protect against SRD disease and enrich academic evidence about SRD disease. **Methods**: The methods used were field surveys and laboratory analysis. Research activities included (1) sampling, (2) isolating the pathogen from symptomatic citrus plants, (3) pathogenicity testing, (4) morphological and molecular identification of the pathogen, (5) DNA amplification, (6) DNA electrophoresis, and (7) DNA sequence analysis. **Results**: The investigation successfully identified Lasiodiplodia theobromae as the pathogen responsible for causing citrus stem rot disease in Bali Province. **Conclusion**: This research provides crucial information about the main pathogens of SRD in Bali, particularly on citrus plants. The findings can serve as an early warning system to prevent the spread of SRD and contribute to the scientific understanding of this disease.

Cite this article:
Wirya, G. N. A. S., Agung, I. D. A. P., & Sudiarta, I. P. (2025). Detection and molecular identification of citrus stem rot disease in Bali Province. *AGROMIX, 16*(1), 1–12. https://doi.org/10.35891/agx.v16i1.4075

INTRODUCTION

Citrus is one of the most significant horticultural commodities, with high demand and profitability. Bali Province has an area of 563,666 hectares, or 0.29% of the area of the Indonesian archipelago, consisting of a rice field area of 407,534 ha and a plantation area of 328,908 ha (BPS Bali Province, 2017). The largest citrus-producing center is in Gianyar Regency, with a total production of 126,101 tons/year. Bangli Regency, with a total production of 104,528 tons/year, followed by other districts such as Badung, with a total production of 3,307 tons/year, Karangasem with a total production of 386 tons/year, and Buleleng's total production of 5,521 tons/year (BPS Bali Province, 2021).

The development of horticultural agricultural areas in Bali Province is mostly mixed with plantation areas. However, several locations are designated for vegetable and fruit crops. Bali citrus has competed as a superior commodity with other imported citrus commodities. Therefore, efforts should be made to enhance both quality and productivity. Between 2017 and 2021, citrus production in Bali Province fluctuated. In 2017, citrus production in Bali Province was 167,513 tons/year, but it went up to 223,448 tons/year in 2018, according to Indonesian Statistics (2021). In 2019, citrus production rose to 349,400 tons/year; in 2020, it increased to 490,090 tons/year; in 2021, it declined to 239,866 tons/year. The annual variations in climate conditions and pest and disease infestations are among the factors that contribute to these fluctuations (Mehmood *et al.*, 2020).

Stem rot disease (SRD), also commonly known as *blendok* in citrus plants, is caused by various species of pathogenic fungi. Liu *et al.* (2022) reported that *Phytophthora spp.*, *Diplodia spp.*, *Botryodiplodia theobromae*, and *Lasiodiplodia theobromae* are some of the fungi frequently responsible for causing stem rot disease (SRD) (Liu *et al.*, 2022). A study by Ismail *et al.* (2012) in Malaysia successfully isolated *Lasiodiplodia theobromae* as the primary causal agent of *dieback* disease in citrus plants, exhibiting symptoms similar to SRD. The identification of *Lasiodiplodia theobromae* as the pathogen causing SRD was also reported by Agung (2019) in Bangli Regency, Bali, based on molecular analysis. However, further research is needed to determine the distribution and prevalence of this pathogen across the Bali Province, considering that diverse agroecosystem conditions may influence the composition of SRD-causing pathogens (Widyastiti & Widodo, 2021). Additionally, recent studies have also indicated the existence

of *Lasiodiplodia* species complexes involved in plant diseases, highlighting the importance of species-level identification for the development of appropriate control strategies (Cruywagen *et al.*, 2017).

In 2019, molecular analysis identified the pathogen responsible for SRD in Bangli Regency as *Lasiodiplodia theobromae.* Symptoms include cracks on the skin's surface at the base or root crown area and the discharge of golden brown sap (gummosis), leading to a rotten and sour smell. The disease's severity is 64% (Agung, 2019). *Phytophthora* spp., *Diplodia* sp., *Botryodiplodia theobromae*, and *Lasiodiplodia theobromae* have a relatively wide host distribution. Therefore, several pathogen species that cause SRD disease in citrus plants exhibit identical symptoms and signs. SRD disease may be caused by *Phytophthora* spp., *Diplodia* sp., *Botryodiplodia theobromae*, or *Lasiodiplodia theobromae* individually or in combination. Hence, molecular study is necessary to identify and ascertain the prevailing pathogen species responsible for SRD disease in Bali Province.

METHODS

Tools and materials

The equipment used were Erlenmeyer, scales, Bunsen lamp, Petri dish, micropipette, autoclave, laminar air flow, scissors, stove, pan, spoon, filter, measuring cup, plastic bag, mask, label, stationery, ruler, camera, laptop, oose needle, cork borer, microscope, optilab, PCR (Polymerase Chain Reaction), Eppendorf, vortex, centrifugation, electrophoresis, and ultraviolet transilluminator.

The materials used were distilled water, 70% alcohol, 0.5% clorox, PDA media (250 g potato, 20 g dextrose, 20 g agar, 1000 mL distilled water), NA media (nutrient agar), liquid nitrogen, CTAB buffer, TE buffer, ITS1 primer, ITS4 primer, mercaptoethanol, sodium acetate, chloroform isoamyl alcohol, ethanol (70%), and samples of diseased citrus plants.

Location

Research was carried out at the Plant Disease Laboratory, study program Agroecotechnology, Faculty of Agriculture, Udayana University. Sampling was conducted in various citrus-producing center districts in Bali Province, namely Gianyar, Badung, Bangli, Buleleng, and Karangasem, between February and May 2023.

Methods

Research activities included surveys and isolation sampling. Observations of disease occurrence were carried out diagonally on a land area of approximately 50 m x 50 m with five sample points, and at each point, 10 plants were selected. For each sample tree, the stem base of the plant exhibiting SRD symptoms was chosen, yielding a total of 50 citrus plant stem bases at the observation site. Subsequently, disease incidence (DI) was determined through observations and calculations using the formula as outlined by Mohammed *et al.* (2000):

$$DI = \frac{\sum \text{symptomatic plants}}{\sum total\ plants}$$

Disease severity (DS) due to SRD pathogens in citrus planting areas was calculated using the formula following Widyastiti & Widodo (2021):

$$DS = \frac{\sum(ni \times vi)}{N \times V} \times 100\%$$

Note:
ni = Total plants based on score
vi = Score based on symptoms
N = Total plants
V = Maximum score in cropping area

Plants were categorized into several scoring categories based on the severity of their symptoms. A scoring system was used to classify SRD disease in citrus during sampling.

Table 1 presents the score assessment criteria based on plant symptoms to evaluate the severity of stem rot disease (SRD) in citrus plants. The scoring system ranges from 0 to 4, where a score of 0 indicates a healthy or asymptomatic plant, while a score of 4 represents a highly impacted tree with widespread infection leading to mortality. This classification helps in systematically identifying the disease progression, from mild to severe symptoms,

based on the percentage of infected branches, canopy condition, and overall plant health. The assessment serves as a crucial tool for monitoring SRD severity and implementing appropriate disease management strategies.

Table 1. Score Assessment Based on Plant Symptoms

Score	Information
0	Healthy or asymptomatic for SRD pathogens
1	Mild symptoms: 25% of branches are infected, the canopy turned yellow, or falls <25%
2	Moderate symptoms: Widespread infection, 25%-50% of branches infected, crown yellowing or falling, ≥ 25 branches drying
3	Severe symptoms: Plants suffer, 50%-75% of branches are infected, and branch death follows.
4	Highly impacted: 75%-100% of the tree is infected leading to tree mortality.

Source: Widyastiti & Widodo (2021)

Inoculum source testing

Samples showing indications of SRD were trimmed at the skin interface between healthy and diseased tissue, measuring 3 cm × 3 cm, and subsequently rinsed with running water to remove any adhering debris. Following that, surface disinfection was performed with 70% alcohol for 1 minute, followed by rinsing with sterile distilled water three times, and then proceeding with planting in PDA media. Fungi were cultivated and purified in the same medium until a pure culture was achieved. The incubation period lasted 3-5 days (Wei et al., 2022; Yadav, 2021; Movet et al., 2015).

Pathogenicity test

Pathogenicity tests were conducted on citrus stems by spraying with sterile water, 0.5% clorox, and rinsing with sterile water. Five punctures were made on the stem surface using a needle. Seven-day-old pure culture pieces from symptomatic plant isolates were placed on the incision, wrapped with cotton soaked in sterile water, and secured with tape. This is intended to promote germination and infection. The development of infection was monitored daily by examining the symptoms that occur, as described by Retnosari (2011). Isolates that exhibit SRD symptoms during the pathogenicity test were then identified based on their morphology.

Morphological identification

Pure cultures of SRD pathogens were identified morphologically by looking at the shape of hyphae, conidia, conidiophores, and culture characteristics on PDA media. The data were cross-referenced with the CMI Descriptions of Pathogenic Fungi and Bacteria book to identify the species.

Molecular identification

The DNA extraction was conducted according to the methods outlined by Doyle and Doyle (1987). A 0.2 g sample of pathogenic fungal mycelium was ground with liquid nitrogen. The pathogenic fungal powder was placed in an Eppendorf tube with 500 µL of CTAB buffer and 50 µL of β-mercaptoethanol. The mixture was then homogenized using a vortex. Cell walls were disrupted by heating at 70°C for 60 minutes, with agitation every 10 minutes to accelerate lysis. Subsequently, the sample was cooled to ambient temperature. Then, 500 µL of isoamyl alcohol chloroform (24:1) was added to the tube, vortexed until well mixed, then centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to a new Eppendorf tube with the addition of 500 µL sodium acetate. It was mixed until uniform by vortexing and centrifuged at 12,000 rpm for 10 minutes. The tube was gently agitated to facilitate DNA binding and then placed in an incubator at -20°C for 30 minutes. The DNA strands were precipitated using centrifugation for 10 minutes. The liquid supernatant was removed, and the solid pellet was rinsed with 70% ethanol before being centrifuged at 8,000 rpm for 5 minutes. The ethanol was disposed of, and the pellet was dried. The pellet was mixed with 50 µL of TE buffer and kept at -20°C for future application in the DNA amplification.

DNA amplification

DNA amplification was conducted using Thermo Cycle PCR equipment. Amplification was conducted using universal primers to identify the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). The forward primer was ITS1 (5'- CTTGGTCATTTAGAGGAAGTAA-3'), and the reverse primer was ITS4 (5'- TCCTCCGCTTATTGATATGC-3'). The resulting amplification produced a fragment of 490 bp. The DNA amplification reaction was carried out with a total volume of 25 µL, consisting of 1 µL DNA, 2.5 µL buffer 10 x and Mg^{2+}, 0.5 µL dNTP 10 mM, 1 µL each primer, 12.5 µL Taq DNA (10 units /µL), and 9.5 µL H_2O. The amplification consisted of predenaturation at 94°C for 3 minutes, followed by 30 cycles. Each cycle included DNA denaturation at 94°C for 1 minute, primer attachment at 45°C for 1 minute, and DNA synthesis at 72°C for 2 minutes. For the last cycle, the synthesis stage was extended by 10 minutes. The cycle concluded at a temperature of 4°C.

DNA electrophoresis

The amplification products were analyzed using Blued electrophoresis with 1% agarose gel (0.5xTris-Borate EDTA/TBE). Electrophoresis was carried out at 100 volts for 28 minutes, and then the agarose gel was incubated in a dye containing ethidium bromide (1%) for 15 minutes, then washed with H_2O for 10 minutes. The electrophoresis results were visualized with an ultraviolet transilluminator. The DNA bands formed due to electrophoresis were documented with a digital camera.

Data analysis

The data was processed using the Microsoft Excel program. Data was presented in tables and graphs regarding certain characteristics that have been determined.

RESULTS AND DISCUSSION

Symptoms of SRD attack on citrus plants in Bali

SRD infestations on citrus plants in Bali exhibit distinctive symptoms, particularly on mature plants where the bark peeled from the base of the stem to the top. Eventually, the skin would shed. The wound discharged a golden brown fluid known as gummosis, and the skin's surface cracked (Figure 1).

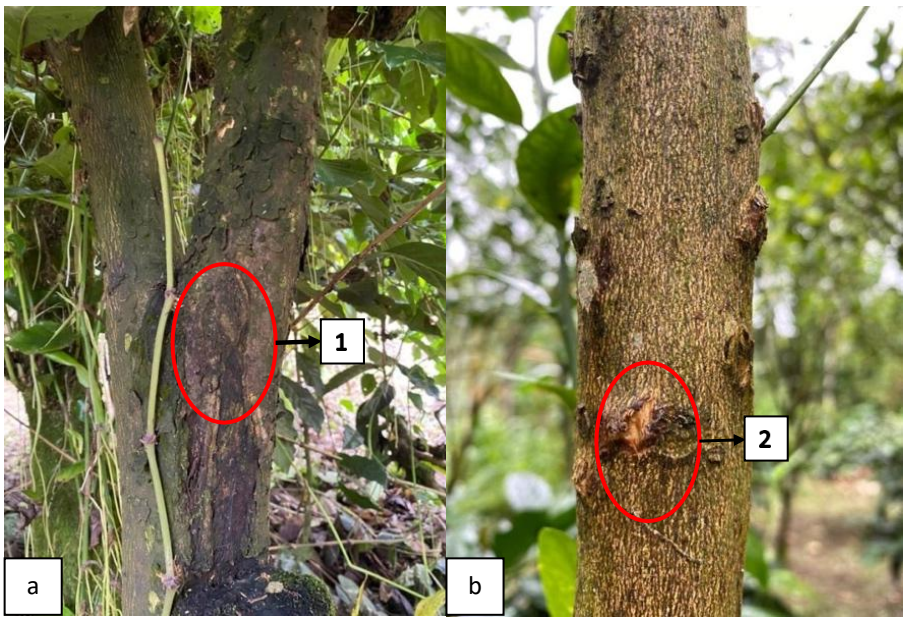

Figure 1. Symptoms of stem base rot on citrus plants: (a) detachment of skin on the stem, (b) rot at the base of the stems, (1) cracks on the stems, (2) rot on the stems

At an advanced stage, symptoms of rot on the surface of the stem may appear limited, but the infection has spread to the cambium, causing the stem tissue to crack, collapse, and release a golden-brown liquid. This finding is supported by Hu *et al.* (2023), who showed that stem rot disease usually causes dryness and subsequently leads to the shedding of plant parts such as leaves, flowers, and fruits (Hu *et al.*, 2023). Stem base rot in citrus plants attacks earlier during the seedling phase, starting from the base of the stem near the soil surface or at the junction between the upper and lower stem. The bark will appear sunken and secrete gummosis, which appears clear when wet but turns golden brown when dry. These symptoms are identical to those observed in citrus plants from Bali infected with the SRD pathogen. If the roots are pulled out, they may appear rotten, leading to plant death (Bodah, 2017) (Ezrari *et al.*, 2022).

In observing symptoms in the field, severe symptoms due to infection with the SRD pathogen resulted in significant defoliation, where twigs and leaves fell off massively, ultimately leading to tree death, which was assigned a severity score of 4. At this stage, the infection had extensively spread, causing irreversible damage to the plant. Symptoms of SRD infection included root rot, decay and darkening of the stems (danckers stems), the discharge of golden-brown fluid (gummosis), progressive twig death (branches dieback), and, in advanced cases, complete plant mortality, categorized with a severity score of 3. Moderate SRD infections displayed a combination of symptoms, including root decay, the appearance of cracks on the stem, gummosis, and noticeable yellowing of leaves, affecting more than 25% of the foliage, which was classified with a score of 2. In contrast, mild SRD infections were characterized by root rot, minor cracks on the stem, the presence of gummosis, and limited yellowing of leaves,

affecting 25% or less of the total leaf area, with a severity score of 1. The progressive development of symptoms at different severity levels is illustrated in Figure 2, demonstrating the gradual impact of SRD infection on plant health and survival.

Figure 2. Symptoms resulting from citrus plant pathogen infection: (a) Yellow Leaves, (b) Death of Branches, (c) Root Rot, (d) Death

The disease incidence rate caused by SRD infection varies from 30% to 50% in each research area. The most severe symptoms were observed in Bayung Gede Village, Bangli Regency, including root rot (danckers stems), gummosis, branch dieback, and plant death. Bayung Gede Village in Bangli had a disease severity rate of 50%. Plaga Village in Badung and Kerta Village in Gianyar had the lowest disease severity scores. The severity level in Telaga Village, Buleleng, was 30%, whereas in Jungutan Village, Karangasem, was 45%. Table 2 depicts the various symptoms and severity levels of SRD on citrus plants in Bali.

Table 2. Types of symptoms and incidence of SRD disease in citrus plants in Bali

Location (Village, Regency)	Symptom Type				Disease Incidence (%)
	Yl	Ds	BD	D	
Pelaga, Badung	-	√	√	-	30
Kerta, Gianyar	-	√	√	-	30
Bayung Gede, Bangli	-	√	√	√	50
Telaga, Buleleng	√	√	√	-	45
Jungutan, Karangasem	√	√	√	-	45

Notes: Yl = Yellow leaves, Ds = *Branch dieback*, Bd = Root rot, D = Death

The development of SRD disease in Bali was very high. Environmental variables are likely responsible for promoting the growth of pathogens. Blanchard and Tattar (1981) stated that disease development is driven by three main factors: the virulent pathogen, the host plant's resistance level, and the environment, known as the three-angle disease.

The five districts have a tropical climate characterized by low air temperatures ranging from 15-30°C and high rainfall, with the lowest average being 7,300 mm and the maximum 36,500 mm/year (BMKG, 2022). Cheng *et al.* (2020) stated that the pathogens *Phytophthora* spp., *Diplodia* sp., *B. theobromae*, or *L. theobromae* develop at low temperatures (15-20°C). The data shows that Bali experiences significant yearly rainfall, which indirectly impacts

humidity levels. This environmental situation facilitates the growth and dissemination of microorganisms responsible for SRD disease.

The severity of SRD disease in citrus plants in Bali is relatively high, ranging from 36.0% to 50.5% (Figure 3). The highest incidence of disease was in Kerta Village, Gianyar, with a DI value of 50.5%, then respectively in Bayung Gede Village, Bangli, with a DI value of 43.5%, and Telaga Village, Buleleng, with a DI value of 38.5%. The lowest disease incidence was in Pelaga Village, Badung, with a DI value of 36.0%.

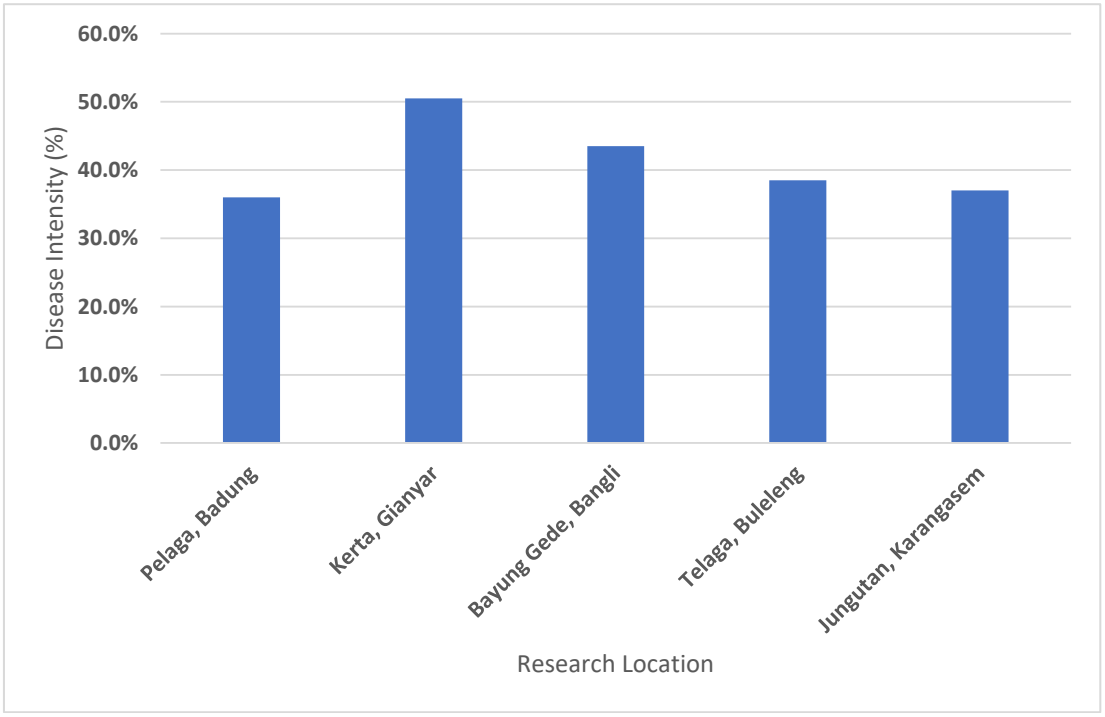

Figure 3. Diagram of SRD Disease Intensity on citrus plants in Bali

Isolation of fungi from citrus plants with SRD symptoms and their pathogenicity

The experiment was conducted on Siamese orange seedlings, the dominant variety grown in Bali Province, which often suffers from SRD disease attacks. Pathogenicity tests showed that fungal isolates that had been inoculated into citrus plantations showed symptoms of SRD 25 days after inoculation. Inoculation of pathogenic fungi on the stem surface shows less pronounced symptoms. However, if the inoculation point is cut, a distinct contrast becomes evident between the treatment and control groups. During the pathogenicity test, the injection of pathogenic fungal isolates resulted in necrotic signs on the surface of the citrus plant stems. Inoculation with sterile water (control) showed no signs of pathogenic mechanisms (necrosis) on the surface of citrus plant stems. Symptoms were more evident when the citrus plant was cut back to the cambium layer, as seen in Figure 4.

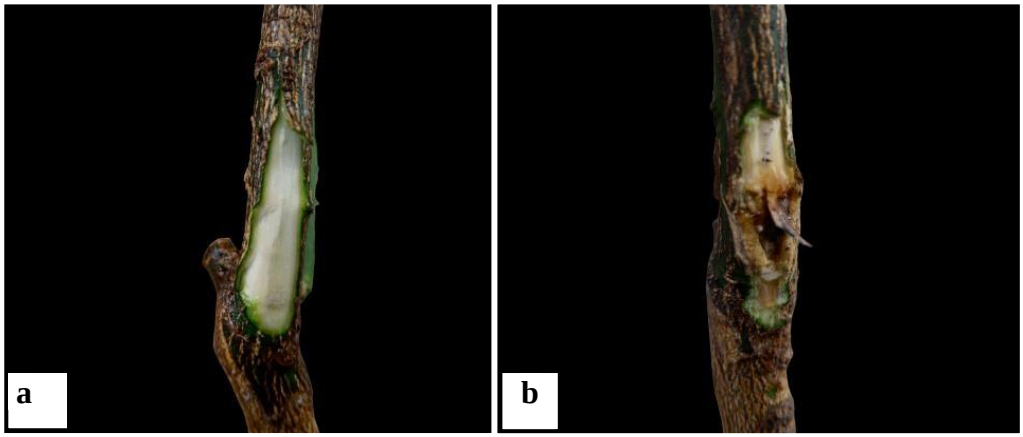

Figure 4. Symptoms of stem base rot resulting from inoculation in the pathogenicity test. (a) No necrotic symptoms were observed after inoculation with sterile water. (b) Necrotic symptoms were observed on citrus stems when pathogen isolates were inoculated.

Sopialena (2017) states that necrosis in citrus plants is caused by pathogen activity, producing cellulolytic enzymes that break down cellulose and hemicellulose in the wood tissue. Cellulose and hemicellulose are degraded into smaller particles that pathogens can utilize, resulting in tissue death. Retnosari (2011) stated that this pathogen can live as an endophyte in plant organs without producing clear symptoms and signs of disease. The disease only appears when environmental conditions are unfavorable for the plant. The symptoms caused by SRD disease in Bali Province by Maryono (2010), who found that the symptoms of SRD disease from inoculation treatment of pathogenic fungi on citrus seedlings showed symptoms of necrosis in the cambium. However, the development of disease symptoms was very slow.

Morphological characteristics of pathogenic fungi isolated from citrus plants with SRD symptoms

Macroscopic identification was carried out by observing the shape and color of the colony. Microscopic observation was used to observe the form of spores and mycelia of pathogenic fungi. Macroscopic observations showed that the fungal isolates, on the third day after being cultured on PDA media, produced the growth of mycelium, which was initially white with abundant growth in the air like fine hair threads or cotton. After the seventh day, the mycelium developed into a grayish white; on the ninth day, the mycelium became black, as shown in Figure 5.

According to Herliyana *et al.* (2019), the pathogenic fungi *L. theobromae* exhibits mycelial proliferation that forms numerous fine threads or cotton-like structures. Colonies initially become gray and subsequently transition to black. The development is rapid, typically occurring within 3-7 DAP on PDA media, generating a circular pattern that covers the entire petri dish.

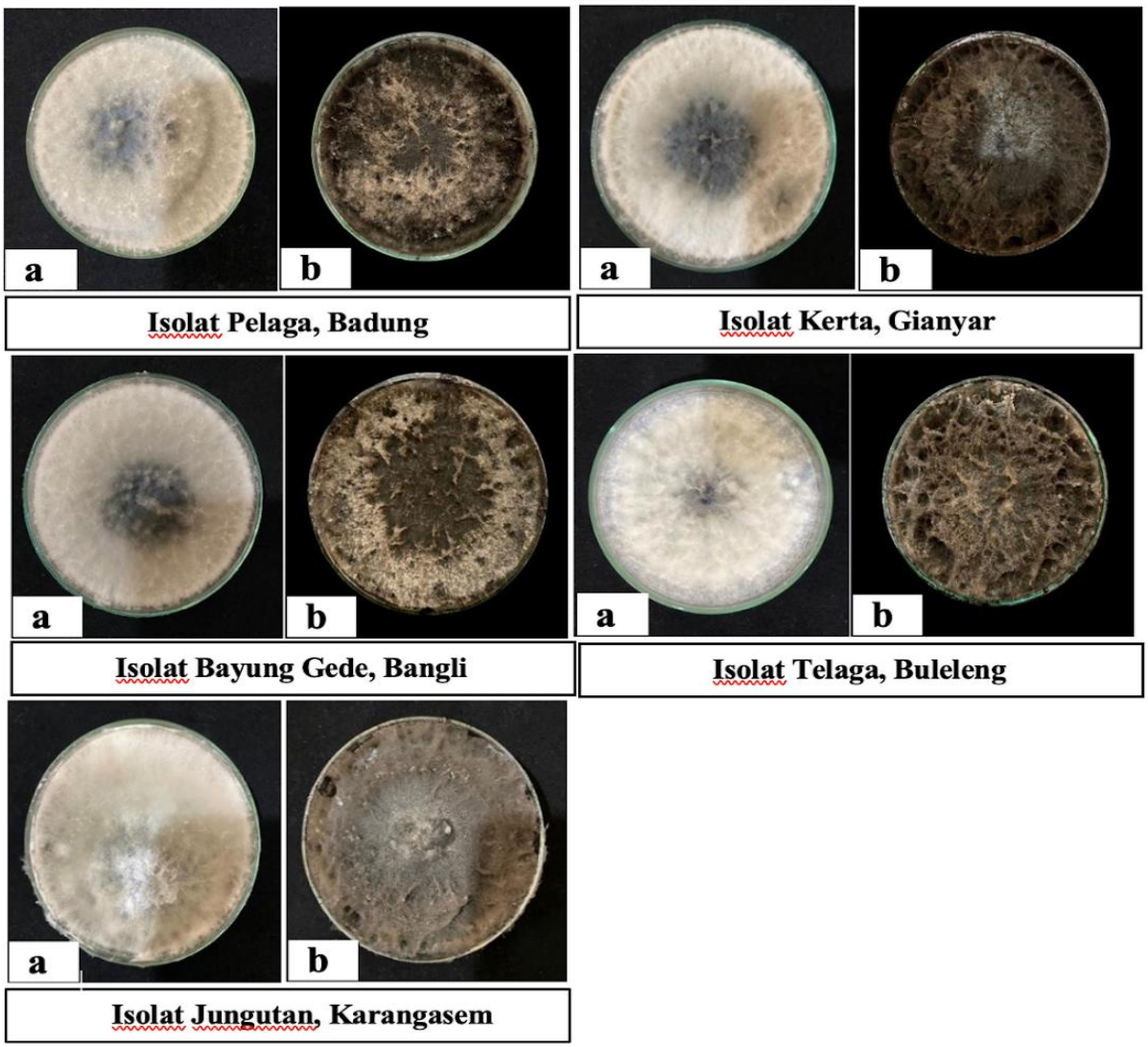

Figure 5. Fungal colonies isolated from citrus plants with SRD symptoms on PDA media at room temperature (25-30°C). (a) Growth of 3-day-old fungi. (b) Growth of 7-day-old fungi.

The macroscopic features align with Rodriguez-Galves *et al.*'s (2017) findings that *L. theobromae* colonies on PDA media had aerial mycelium, which was white at first, then changed to gray, and then black as the colony aged. On PDA

media, isolates from Plaga Village (Badung) grew greyish white with a black color on the reverse. The second isolate from Kerta Village (Gianyar) grew white with black on the reverse. The third isolate from Bayung Gede Village (Bangli) grew grayish white and turned black. The fourth isolate from Telaga Village (Buleleng) grew white and turned black. The fifth isolate from Jungutan Village (Karangasem) grew grayish-white and turned black.

Microscopic identification showed hyphae that were insulated and hyaline in color, equipped with young conidia (microconidia) and mature conidia (macroconidia), found evenly distributed among the hyphae. Microconidia were oval, non-separated, and in groups. Macroconidia were oval-shaped, insulated, and not in groups (Figure 6).

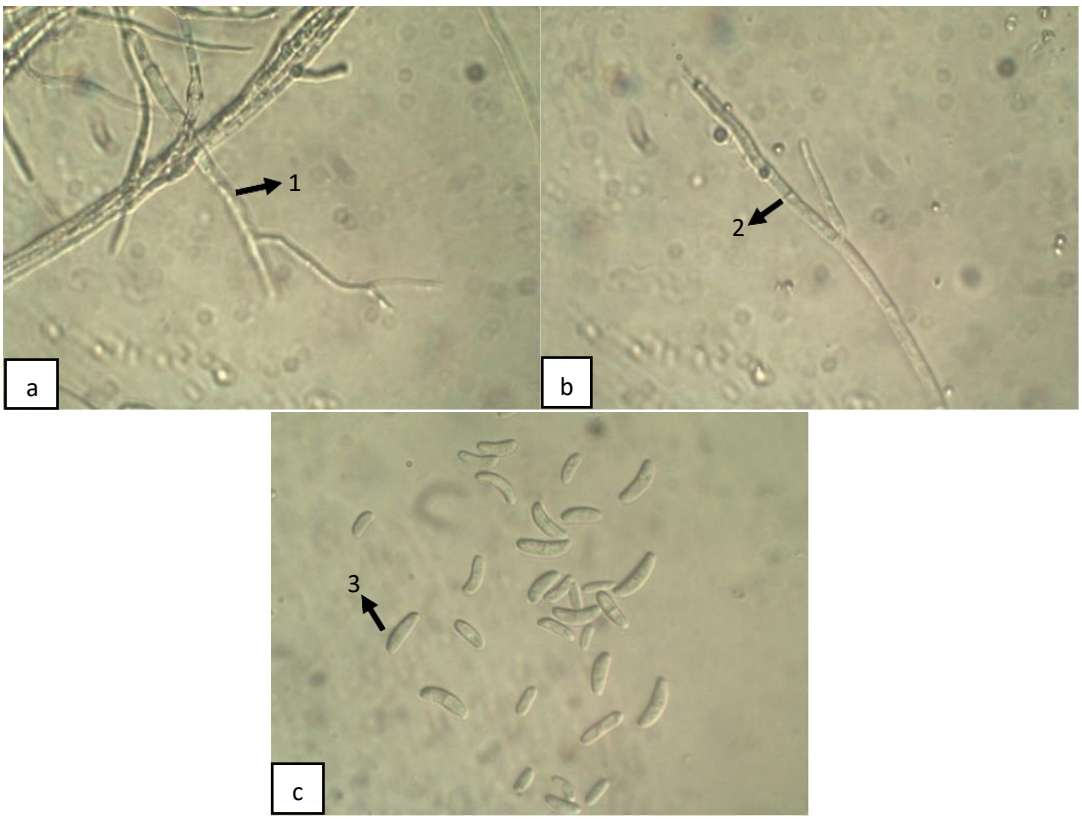

Figure 6. Morphology of spores and hyphae of pathogen isolates, microscopic observation. (a) hyphae. (b) septum in hyphae. (c) young conidia. (1. hyphae, 2. partitions on hyphae, 3. young conidia)

The pathogenic fungal isolates obtained matched the findings of Febbiyanti (2017), which stated that *L. theobromae* has microconidia and macroconidia with different shapes. Microconidia do not have partitions (single-celled), or hyaline, with relatively thick conidia walls. Meanwhile, macroconidia are dark brown, have partitions (two cells), and the thickness of the conidia walls is not visible.

Retnosari (2011) stated that the pathogenic fungi *B. theobromae* have microconidia and macroconidia, both of which are oval. Microconidia are hyaline; their walls consist of two layers and are not partitioned, while macroconidia are brown; their cell walls are only one layer and have one partition, forming two cells. *Diplodia* sp. has hyaline microconidia and is nonseptate or has no partitions, while macroconidia appear hyaline, dark in color, and have one partition.

The macroscopic and microscopic morphological observations were compared with the CMI Descriptions of Pathogenic Fungi and Bacteria and relevant research literature on the symptoms or pathogens associated with SRD disease. The results resemble the pathogens *Diplodia* sp., *B. theobromae*, and *L. theobromae*. These three fungi have similar morphological characteristics and are very difficult to distinguish. To validate the species of the pathogen, investigations continue to molecular identification.

Molecular SRD detection with PCR

The detection of SRD (Stem Rot Disease) was conducted using the PCR method to confirm the presence of the pathogen. Amplification was performed using the forward primer ITS1 and the reverse primer ITS4, which are commonly used for fungal identification based on the ITS (Internal Transcribed Spacer) region. The results of gel electrophoresis (Figure 7) showed that DNA fragments of the SRD pathogen were successfully amplified in all tested samples. A single distinct band of approximately 500 bp was observed in lanes 2, 3, 4, and 5, indicating successful

amplification. The presence of this band across all samples suggests that the pathogen is consistently associated with the tested plants. A molecular size marker (DNA ladder) was used in the first lane to estimate the fragment size, confirming that the amplified product corresponds to the expected size of ±500 bp. These findings provide molecular evidence of SRD pathogen infection in the tested samples, supporting the identification of the causative agent through genetic analysis.

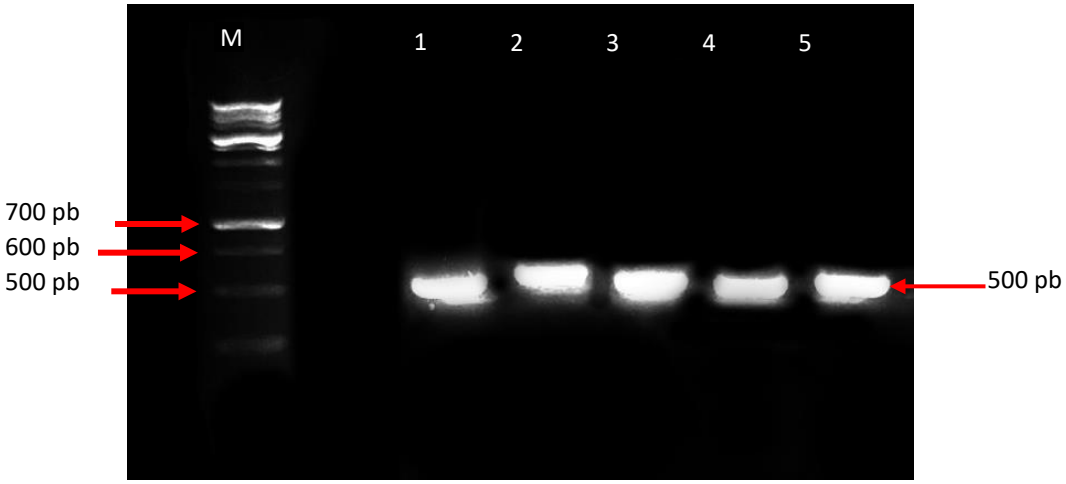

Figure 7. Visualization of PCR results from citrus plant fungi isolates with SRD symptoms using 1% agarose gel. M= Marker (Smobio 1 kb ladder); 1 = Karangasem; 2 = Badung; 3 = Bangli; 4 = Gianyar; 5 = Buleleng

Ashokkumar *et al.* (2018) used forward primer ITS1 and reverse primer ITS4 to differentiate species of the genus Lasiodiplodia that could not be differentiated based on morphological characters. The PCR technique is employed in the ITS region due to the broad spectrum of this sequence, which enables the identification of numerous pathogenic fungi. The DNA amplification process using primers ITS1 and ITS4 included three open reading frames (ORF), namely ITS1, ITS4, and the 28 s rDNA gene. The results of the ITS gene amplification, which was carried out using ITS1 and ITS4 primers, were followed by sequencing to determine the DNA sequence, then looking for homology and relationship of the DNA in the GenBank database. Adesemoye *et al.* (2014) successfully amplified the ITS gene from the fungi *L. theobromae* using primers ITS1 and ITS4 with a band size of 550bp.

Phylogenetic sequencing and analysis

The sequence results were compared to various GenBank (NCBI) DNA sequences to identify similarities. The sequencing results indicated that fungal isolates obtained from citrus plants with SRD symptoms in Bali had strong nucleotide sequence similarities, ranging from 95% to 100% (Table 3).

Table 3. Homology (%) of nucleotide sequences among *L. theobromae* isolates that infect fruit plants worldwide

ISOLATES	Karangasem	Badung	Bangli	Gianyar	Buleleng
KU291531.1 *L. theobromae (*ECUADOR)	98%	96%	100%	100%	98%
OL782125.1 *L. theobromae* (THAILAND)	98%	96%	100%	100%	98%
MK530072.1 *L. theobromae* (MALAYSIA)	98%	96%	100%	99%	98%
OP095032.1 *Lasiodiplodia* Sp. (INDIA)	98%	96%	100%	99%	98%
KU377509.1 *L. theobromae* (VENEZUELA)	98%	96%	100%	100%	98%
MF136589.1 *Diplodia* Sp. (USA)	87%	86%	88%	88%	87%
MH863765.1 *Diplodia* Sp. (RUSSIA)	88%	87%	89%	89%	88%
MK625223.1 *Diplodia* sp. (ITALY)	86%	86%	88%	88%	87%
KT240354.1 *Diplodia* sp. (SPANYOL)	87%	86%	88%	88%	87%
AY259091.1 *Botryosphaeria lutea* (PORTUGAL)	82%	82%	84%	84%	82%

The nucleotide sequence showed 100% similarity with *L. theobromae* strains from Ecuador, Thailand, Malaysia, India, and Venezuela. The kinship analysis indicates that the sequence homology of the five isolates from Bali province closely resembles the pathogenic species *Lasiodiplodia* sp. and *Lasiodiplodia theobromae*. Sathya *et al.* (2017) described the *Lasiodiplodia* species as a globally distributed pathogen in tropical and subtropical regions. It infects over 280 plant species, causing a variety of diseases. It causes dieback and cancer, particularly in woody plants (Alejandra *et al.*, 2014).

Cheng *et al.* (2020) state that SRD disease is caused by the pathogenic fungi *L. theobromae*, a synonym of *B. theobromae*. The genus Lasiodiplodia is reported to have a sexual or teleomorphic phase, depending on the species. During the teleomorph phase, *Lasiodiplodia theobromae* is known as *Botryosphaeria rhodian*. Zhang *et al.* (2006) identified Diplodia, Lasiodiplodia, Neofusicoccum, Pseudofusicoccum, Dothiorella, and Sphaeropsis as members of the Botryosphaeriaceae group, pathogenic fungi that contain several species spread across several anamorph genera. There have been 224 species of Lasiodiplodia identified since 1884. *L. theobromae* is the dominant species of the Lasiodiplodia genus, a pathogen in several woody plants, especially in tropical areas (Salvatore *et al.*, 2020).

According to Widyastiti (2017), *L. theobromae* is the primary pathogen affecting citrus plants in many regions of Indonesia, particularly in Bangli Regency, causing rot symptoms at the stem's base. The pathogenic fungi *L. theobromae* has a broad host range and can infect several crop commodities besides citrus. Infections from different plants cause varying symptoms on different host plants, such as decreased plant growth leading to death in guava plants (*Psidium guajava* Linn.) (Safdar *et al.*, 2015), shoot dieback in sisham plants (*Dalbergia sissoo* Roxb.) and grape plants (*Vitis vinifera* L.) (Shah *et al.*, 2014). Meanwhile, the pathogenic fungi *L. theobromae* on castor oil plants (*Jatropa curcas* L.) causes rot at the roots and base of the stem (Liu *et al.*, 2022). The nucleotide sequence data was reanalyzed using phylogeny to establish the link between the pathogenic fungi.

The five DNA samples were sent to Macrogen, Singapore. The SRD isolate sequence was ±500 bp long. Analysis of the sequencing results with ClustalW multiple alignments in Bioedit to determine conserved areas in the sequence. An alignment sequence identity matrix was used to determine the homology between sequences. The homology analysis of 5 SRD sequences from Bali isolates collected from various SRD-infected citrus plants revealed similarities ranging from 96% to 100%. The phylogeny was carried out from the sequence results that had been aligned previously and then entered into the Clustal.

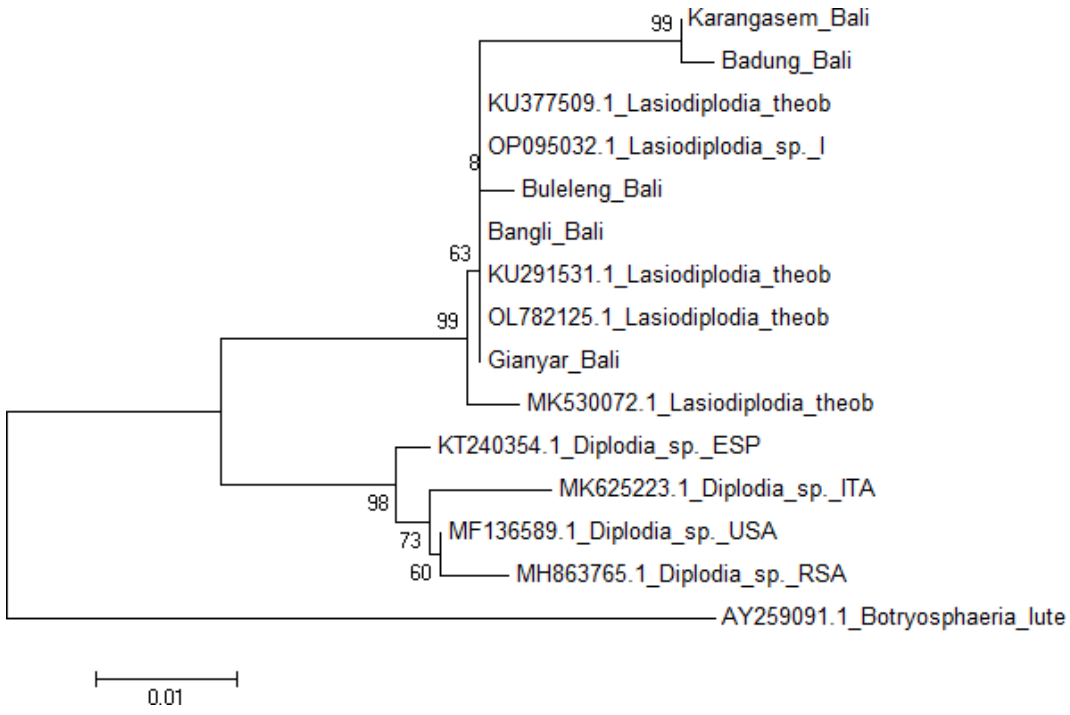

Figure 8. Phylogenetic tree of pathogen isolates that have similar species to isolates from several countries (Ecuador, Thailand, Malaysia, India, Venezuela, USA, Russia, Italy and Spain). The pathogenic fungi Botryosphaeria lutea (Portugal) was used as a comparison.

The five isolates had a close relationship with the pathogen isolate *Lasiodiplodia theobromae*. It was shown in the Karangasem and Badung isolates, which had the closest relationship to isolates from Venezuela (*L. theobromae*). Bangli, Gianyar, and Buleleng isolates have the closest relationship with isolates from Ecuador and Thailand (*L. theobromae*). *Diplodia* sp. is in the same family as *Lasiodiplodia theobromae* and has a distant relationship with the

five isolates isolated from citrus plants in Bali. It indicates that the five isolates isolated in Bali are closely related to the pathogenic species *Lasiodiplodia theobroamae*.

CONCLUSION

This study successfully identified morphologically and molecularly that Lasiodiplodia theobromae is a pathogen that causes stem rot disease (SRD) in citrus plants in Bali Province. Analysis of five isolates obtained from various citrus production centers in Bali showed that all of them were Lasiodiplodia theobromae species. These results confirm that L. theobromae is the dominant pathogen that causes SRD in citrus in Bali. Given that L. theobromae has a wide host range and can cause various diseases in other plants, further research is needed to understand the source of inoculum and the spread of this pathogen in the Balinese agricultural ecosystem. In addition, further research also needs to focus on developing effective and sustainable SRD control strategies, including the use of citrus varieties that are resistant to L. theobromae and the implementation of healthy cultivation practices.

ACKNOWLEDGMENT

We sincerely express our gratitude to the Head of the Plant Disease Laboratory at the Faculty of Agriculture, Udayana University, for their exceptional guidance and unwavering support, which significantly contributed to the success of this research. Their expertise and dedication have been invaluable throughout the process.

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