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MOLECULAR IDENTIFICATION OF PATHOGENS CAUSING ANTHRACNOSE DISEASE IN CHILI FRUIT (*Capsicum frutescens* L.) IN BALI PROVINCE

By:

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Abstract

Chili fruit found both in plantations and in markets, we often encounter anthracnose disease that damages chili fruit. The appearance of sick chili fruit is very disturbing for consumers to buy and use as a cooking flavouring. Molecular identification to determine the cause of the disease was first carried out in Bali Province, as a form of ensuring the species of fungus that damages chili fruit. The results of the study showed that fungal isolates were detected using the PCR method. Amplification was carried out with the forward primer ITS1 and reverse primer ITS4 to confirm the fungus in chili plants. Fungal DNA fragments were successfully amplified from all samples with a size of ±500 bp. The amplified DNA samples were then used for the sequencing stage to determine the fungal species. Sequencing analysis confirmed that the fungal sample on chili was the *Colletotrichum capsici* species with 99.6- 100% homology to several *Colletotrichum capsici* isolates in genebank.

Keywords:

Molecular; PCR; chili; *Colletotrichum capsici*; DNA.

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INTRODUCTION

Chili peppers can be used as a seasoning because of their spicy taste, while chili production in Bali Province in 2020 was 43,380 tons, in 2021 it was 40,922 tons, while in 2022 it was 34,948 tons [1]. Chili production is decreasing every year, one of the causes is plant disease.

There are several diseases that cause loss of cayenne pepper fruit yield, including: bacterial soft rot (*Erwinia carotovora*), gray mold (*Botrytis cinerea*), Rhizopus rot (*Rhizopus stolonifer*), watery soft rot (*Sclerotonia sclerotiorum*), black mold (*Alternaria alternata*), anthracnose (*Colletotrichum capsici*), and sour rot (*Geotrichum candidum*) [2]. According to Sudarma (2020) [3] there are 12 types of diseases that affect chili plants. These diseases include: Anthracnose, Cercospora leaf spot, Choanephora blight, damping off and root rot, Fusarium wilt, fungal fruit rot, gray leaf spot, gray mold, Phytophthora blight, powdery mildew, stem rot and Verticillium wilt.

Anthracnose in chili plants can cause serious problems for farmers worldwide. The disease has been reported in Florida, United States. In Korea, the disease has been reported to be caused by *C. gloeosporioides* (Penz.) Penz. & Sacc.[4]. The disease was first reported in 1890 from New Jersey, USA by Halsted, who described the causative agents as *C. piperatum* and *C. ningrum.* These taxa were later considered as synonyms of *C. gloeosporioides* by von Arx in 1957 [5].

Anthracnose disease is one of the major economic constraints to world chili production, especially in tropical and subtropical regions. Accurate taxonomic information is needed for effective disease control. In the Colletotrichum pathology system, different species of *Coletotrichum* can be associated with chili anthracnose although several species of Colletotrichum have been reported as causative agents of chili anthracnose disease worldwide [6].

MATERIALS AND METHODS

Disease Study

Sick chili fruits were collected and photographed to determine the symptoms of the disease, then the sick fruit was taken to the laboratory to be isolated to determine the cause of the disease. Sick fruit was cleaned with 70% alcohol soaked for 2 minutes, then cleaned with sterile water. Slices of sick chili (the border between sick and healthy) were grown on a Petri dish previously filled with PDA. For 3 days, it was isolated again on a Petri dish to ensure its purity. Furthermore, a pathogenicity test was carried out.

Molecular Identification

DNA extraction followed the procedure of Doyle and Doyle (1987) [7], A sample of 0.2 g of pathogenic fungal mycelium was ground with liquid nitrogen and the pathogenic fungal powder was put into an Eppendorf tube. Then 500 µL of CTAB buffer and 50 µL of β-mercaptoethanol were added, then mixed until homogeneous with a vortex. To lyse the cell

wall, heating was carried out at a temperature of 70ºC for 60 minutes where every 10 minutes it was turned over to accelerate the lysis process. Then cooled to room temperature. Then 500 µL of chloroform isoamylalcohol (24:1) was added to the tube and then mixed until homogeneous with a vortex and centrifuged at 12,000 rpm for 15 minutes. The supernatant obtained was transferred to a new eppendorf tube by adding 500 µL of sodium acetate mixed until homogeneous with a vortex and centrifuged again at 12,000 rpm for 10 minutes. The supernatant was transferred to an eppendorf tube then added 500 µL of sodium acetate and isopropanol each, mixed until homogeneous with a vortex and centrifuged again at 12,000 rpm for 10 minutes. The tube was shaken gently to bind the DNA and incubated at -20 ºC for 30. minutes. The DNA strands obtained were precipitated by centrifugation for 10 minutes. The supernatant was discarded, the pellet was washed with ethanol (70%) then centrifuged at 8,000 rpm for 5 minutes. Ethanol was discarded and the pellet was dried. The pellet was resuspended with 50 µL of TE buffer and stored at -20ºC for further use in the DNA amplification process.

DNA Amplification

DNA amplification was performed on a Thermo Cycle PCR machine. Amplification was performed using universal primers to detect the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA), namely forward primer ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') with a target size of 490 bp (Doyle and Doyle, 1987). The DNA amplification reaction was performed with a total volume of 25 µL consisting of 1 µL of DNA, 2.5 µL of 10 x buffer and Mg2+, 0.5 µL of 10 mM dNTP, 1 µL of each primer, 12.5 µL of Taq DNA (10 units/µL), and 9.5 µL of H2O. The amplification conditions are divided into several stages, namely predenaturation 94 ºC for 3 minutes, followed by 30 amplification cycles, each cycle consisting of DNA thread separation/denaturation 94 ºC for 1 minute, primer attachment/annealing 45 ºC for 1 minute, DNA synthesis 72 ºC for 2 minutes. Especially for the last cycle, a synthesis stage for 10 minutes is added, then the cycle will end at a temperature of 4 ºC.

DNA Electrophoresis

The amplified 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 H2O for 10 minutes. The electrophoresis results were visualized with an ultraviolet transilluminator. The DNA bands formed in the electrophoresis results were documented with a digital camera.

DNA Sequence Analysis

The amplification product was sent to 1st Base (Malaysia) for nucleotide sequencing. The sequencing results were then analyzed using the basic local alignment search tool (BLAST) program with an optimization program to obtain DNA base sequences that have homology with DNA sequences found on the National Center for Biotechnology Information

(NCBI) website. The obtained nucleotide sequences were then analyzed using ClustalW multiple alignment on the Bioedit sequence alignment editor software version 7.0.5. The homology results approaching 100% similarity were categorized as the same species as the sample species.

RESULTS AND DISCUSSION

Disease Study

The results of the study showed that disease symptoms were found in diseased fruit with symptoms of rotting, curved, dry and brown (Figure 1A), compared to healthy fruit (Figure 1B), then the results of isolation on the Petri dish found white cotton-like fungal mycelium that grew a little slow to fill the Petri dish (Figure 1C). The results of observations under a light microscope found oval-shaped fungal conidia measuring 1-3 x 4-6 µm.

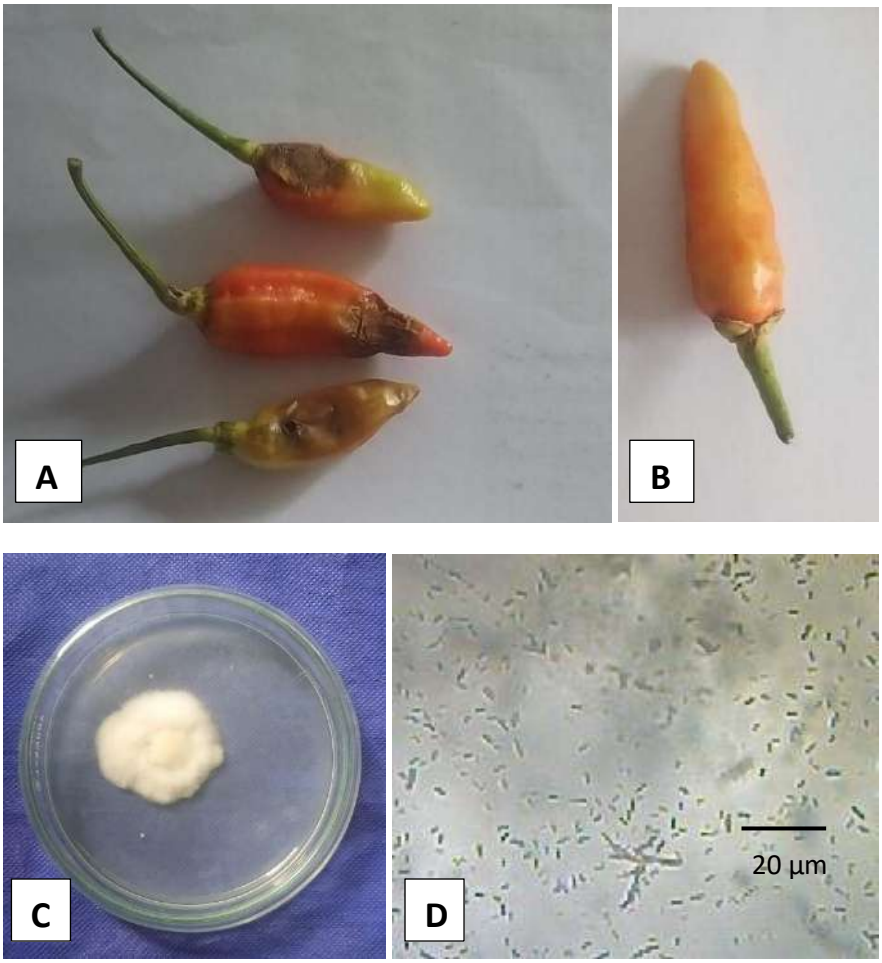

Figure 1. Study of anthracnose disease. (A= symptoms of anthracnose disease on fruit, B= healthy fruit, C= fungal mycelium on Petri dish and D= conidia of anthracnose pathogen)

Anthracnose, derived from the Greek word meaning ‘coal’, is a common name for plant diseases characterized by very dark, sunken lesions containing spores [8]. In general, anthracnose disease is caused by Colletotrichum species belonging to the Kingdom Fungi; Phylum Ascomycota, Class Sordariomycetes; Order Phyllachorales; and Family Phyllachoraceae. Its anamorph is the Glomerella species. Chili anthracnose was first reported

from New Jersey, USA, by Halsted (1890) [25) in 1890 who described the causative agents as *Gloeopsorium piperatum* and C. nigrum. These taxa were later considered synonyms of *C. gloeosporioides* by von Arx (1957) [9].

Anthracnose causes severe pre- and post-harvest damage to chili fruits causing anthracnose lesions. Even small anthracnose lesions on chili fruits reduce their market value [10]. Many post-harvest diseases of fruits exhibit a phenomenon where symptoms do not develop until the fruit is ripe. Colletotrichum species are the most important pathogens causing latent infections [11]. Appressoria are known to form adhesive discs that adhere to the plant surface and remain latent until physiological changes occur in the fruit [12].

Accurate identification of Colletotrichum species together with knowledge of the populations responsible for an epidemic is essential for developing and implementing effective disease control strategies [13]. Traditionally, identification and characterization of Colletotrichum species has been based on morphological characters, such as size and shape of conidia and appressoria; presence of setae; teleomorphic state and cultural characters such as colony colour, growth rate and texture [14]. These criteria alone are not always sufficient for species identification due to overlapping morphological characters and phenotypic variation among species under different environmental conditions. Conidial shape has been applied as a reliable means of distinguishing certain species; for example, conidial shape has distinguished Colletotrichum species pathogenic to strawberries [15], however, in other cases, identification can be complicated due to overlapping conidial morphologies and variation in colony characteristics.

Molecular Detection of Fungal Isolates by PCR

Detection of fungal isolates was carried out using the PCR method. Amplification was carried out with the forward primer ITS1 and reverse primer ITS4 to confirm the fungus in chili plants. Fungal DNA fragments were successfully amplified from all samples with a size of ±500 bp (Figure 2). All five samples were amplified 500 bp.

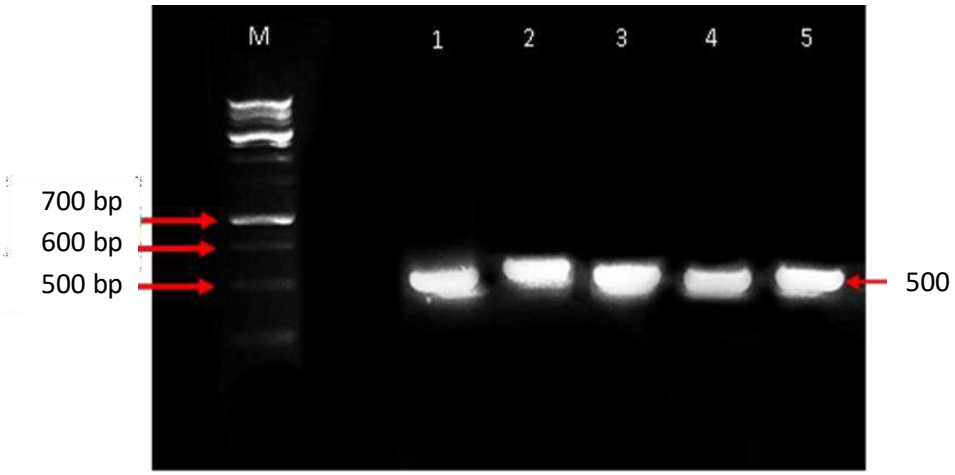

Figure 2. Visualization of PCR results from fungal isolates on chili using 1% agarose gel. M= Marker (Smobio 1 kb ladder); K-= Negative control (ddh2o), 1 = Control + (fungal isolates from lab collection); 2-5 = Fungal isolates on chili repeated 4 times.

Molecular Characterization of Fungal Isolates

Fungal Isolates (*Colletotrichum capsici*)

A 500 bp DNA fragment was successfully amplified from the fungal isolate using the universal primer ITS1/ITS4 (Figure 1). The amplified DNA sample was then used for sequencing to determine the fungal species. Sequencing analysis confirmed that the fungal sample on chili was the Colletotrichum capsici species with 99.6-100% homology to several *C. capsici* isolates in genebank (Table 1).

Table 1. Homology (%) of nucleotide sequences of C. capsici isolates with several isolates that have been reported in GenBank.

Sikuen Gene Bank	**Homology (%)** ***Colletotrichum capsici*** **_Bali**
JX867217_CHI	99,8
EF016300_CHI	99,8
EF016303_CHI	99,6
EF016299_CHI	99,7
MH367525_CHI	100
EF016301_CHI	99,8
EF016302_CHI	99,9
EF016297_CHI	99,9
LC490867_Lasiodiplodia_theobromae_IDN	69,9

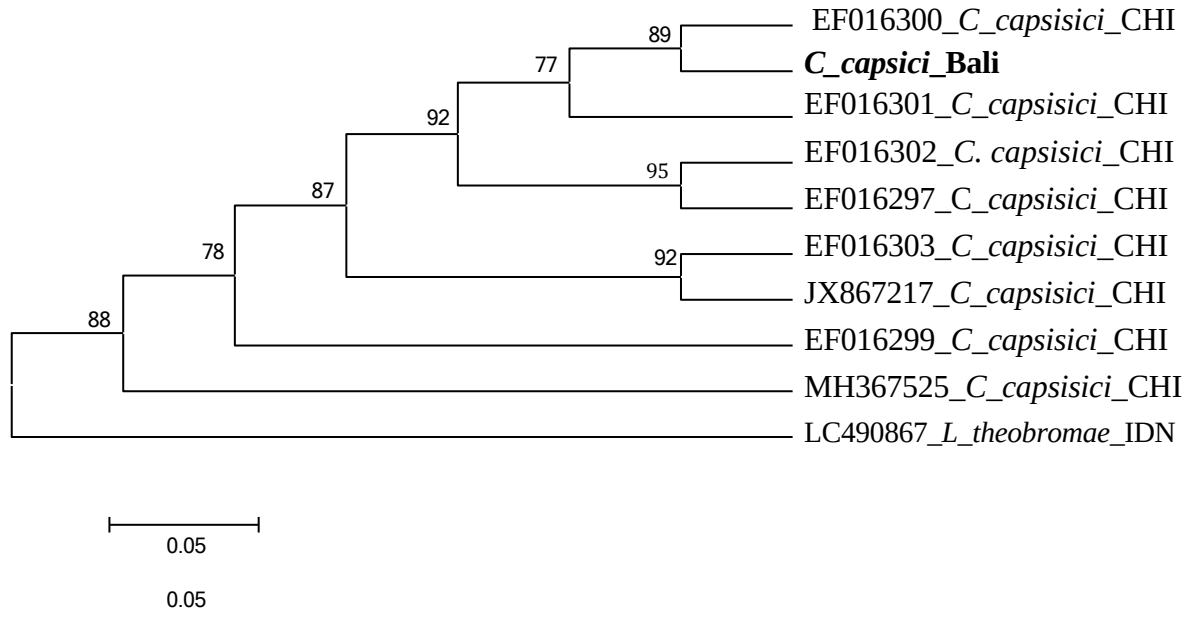

Figure 3. Phylogenetic analysis of Colletotrichum capsici isolates based on partial nucleotide sequence alignment of DNA-A using Mega 6.06 (Neighbor Joining Algorithm with 1,000 bootstraps replicates)

Anthracnose disease in chili is caused by *Colletotrichum* spp. only two have been identified; *C. simmondsii* (previously called *acutatum*), *C. trumcatum* (previously called *capsici*) [24].

Chili anthracnose is caused by *Colletotrichum* species mostly related to the *acutatum*, *truncatum* and *gloeosporioides* complexes. Since 2009 the taxonomy of Colletotrichum has been extensively revised based on multigene phylogenetics, which has had a major impact on the number of species known to cause anthracnose disease in chili. This review discusses (i) the taxonomy of *Colletotrichum* spp. infecting chili, and (ii) the impact of Colletotrichum pathotypes on breeding for anthracnose resistance. To date, 24 Colletotrichum species have been identified as pathogens of chili anthracnose, with the three main pathogens being *C. scovillei, C. truncatum* and *C. siamense*. Several pathotypes have been identified within these three Colletotrichum species, particularly those that can overcome resistance in related *Capsicum* species, *Ca. chinense* and *Ca. baccatum*, will be of major interest to plant breeders as they develop resistant chili genotypes from the transfer of resistance genes from this *Capsicum* species to *Ca. annuum*. Accurate identification of the Colletotrichum species causing anthracnose and a better understanding of the biology of Colletotrichum species and their interactions with hosts will enable better implementation of integrated disease management techniques [16].

In order to overcome the shortcomings of traditional morphology-based identification schemes, DNA sequence analysis has been used to characterize and analyze the taxonomic complexity of Colletotrichum [5, 17, 18, 19, 20). Cannon *et al.* (2000) [21] stated that data obtained from nucleic acid analysis should provide the most reliable framework for constructing a Colletotrichum classification, since DNA characters are not directly influenced by environmental factors. Most fungal phylogenetic studies have used sequences from the ribosomal gene cluster, since these sequences are present in large numbers as tandem repeats and have evolved as a single unit [22]. In particular, sequence analysis of the internal transcribed spacer (ITS) regions located between the 18S and 5.8S genes and the 5.8S and 28S genes, has proven useful in studying the phylogenetic relationships of Colletotrichum species due to their comparative variability [20, 23].

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