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ISOLATION AND IDENTIFICATION OF ANTHRACHNOSE PATHOGEN ON APPLES *(MALUS DOMESTICA* BORKH)

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ABSTRACT

Anthracnose disease in post-harvest apples is very disturbing to consumers, especially the people on the island of Bali, where most of the time during religious ceremonies always use fruit as an offering material. Anthracnose is a disease that damages fruit with symptoms of brown to black spots on the skin of the fruit which can reduce the quality of the fruit. PCR analysis results found 650 bp sized fragments which were successfully amplified from 6 mushroom samples using ITS/ITS4 universal primers. The amplified DNA sample is then used for the sequencing stage, to determine the fungal species. The results of the sequence analysis confirmed that the identity of the fungus in the sample was *Colletotrichum gloeosporioides* with 78-100% homology respectively to several C. *gloeosporioides* isolates and out group in the genebank.

KEYWORDS

identification, molecular, sequence, PCR, anthracnose & *Colletotrichum gloeosporioides.*

INTRODUCTION

Anthracnose disease in apples is caused by *C. gloeosporioides*, one of the causes of post-harvest rot, which results in damage and abnormalities in the appearance of apples that damages the appearance when used as offerings for religious ceremonies in Bali. The genus Colletotrichum comprises some of the most economically important fungi and damaging plant pathogens, such as *C. acutatum* (Simmonds), *C. fragariae* (Brooks), *C. gloeosporoides* (Penz) and *Glomerellacingulata* (Stoneman), the cause of anthracnose in fruit and apple trees. Apple anthracnose is an important disease that inhibits the quality and texture of apples worldwide.*Colletotrichum* spp. cannot be differentiated only by symptoms and signs in the infected tissue. Identification of pathogens is based on morphological characters. Pathogens consist of living filaments known as hyphae, or collectively as mycelium. Pathogens reproduce by spores which may be sexually or asexually. *Colletotrichum* spp. is a major cause of plant diseases, accounting for perhaps 70% of all major plant diseases and is also known as a post-harvest pathogen. Some of these plant pathogenic fungi are called biotrophic because they establish an elaborate feeding relationship with living host cells. Other pathogens are called necrotrophic, because they attack plant tissues aggressively, killing host cells for nutrients (Bajpai *et al.*, 2009).

Bitter rot (anthracnose) is one of the main diseases of post-harvest apples, and is mainly caused by *C.gloeosporioides*. The symptoms of this disease are round, large, necrotic lesions. The edges of the lesion appear slightly higher than the center, where a mass of conidia is produced. As a result, the fruit rots; however, symptoms of infection vary among different hosts. This pathogen can affect leaves at any stage of development, new branches, flowers and fruit. During the rainy season, new branches dropping is the most common symptom; the branches turn purple, then dark brown, dry and brittle (Onofre and Antoniazzi, 2014).

Bitter rot, caused by *C. gloeosporioides* and *C. acutatum*, is an important summer rot disease of apples and pears in Illinois and other apple and pear growing areas. *Glomerellacingulata* is the sexual stage of *C. gloeosporioides*. *Colletotrichum* spp. It also causes leaf spot and canker. More than 50% of fruits are caused by *Colletotrichum* spp. has been observed in several cultivars in several gardens in Illinois (Babadoost, 2015). Bitter rot is the most common apple fruit rot in Kentucky. Commercial and residential crops can suffer devastating losses. Farmers consider bitter rot to be the most important fruit rot and the second most damaging disease in Kentucky apple orchards. Yield losses can range from 10% to 100% (Leonberger*et al.* 2019).

MATERIALS AND METHODS

Place and time of research

The research was carried out in two places: 1) looking for sick and healthy fruit specimens from Batubulan and supermarkets. 2) Laboratory of Plant Diseases and Agricultural Biotechnology Laboratory. The research was carried out from April to August 2021.

Disease Studies

Observing the symptoms of the disease was carried out both macroscopically and microscopically to see the morphology of the pathogen spore structure. Microscopically the pathogen was seen with the help of Optilab to see the pathogen directly through a microscope.

Moleculer Identification

a. DNA extraction

DNA extraction followed the procedure of Doyle and Doyle (1987). A sample of 0.2 g of pathogenic fungal mycelium was crushed with liquid nitrogen and the pathogenic fungal powder was put into an Eppendorf tube. Then 500µl CTAB buffer and 50µl β-mercaptoethanol were added, then mixed until homogeneous with a vortex. To lyse the cell wall, heating was carried out at 70°C for 60 minutes, which was alternated every 10 minutes to speed up the lysis process. Then cooled until it reaches room temperature. Then 500µl of chloroform isoamylalcohol (24:1) was added to the tube and 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 500µl 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 and then 500µl of sodium acetate and isopropanol were

added, mixed until homogeneous with a vortex and centrifuged again at 12,000 rpm for 10 minutes. The tube is shaken gently to bind the DNA and incubated at -20 ºC for 30 minutes. The DNA threads 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. The ethanol was discarded and the pellets dried. The pellet was resuspended with 50µl TE buffer and stored at -20ºC for further use in the DNA amplification process.

b. DNA amplification

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

c. DNA electrophoresis

The amplified product was 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 on the electrophoresis results were documented with a digital camera.

d. DNA Sequence Analysis

The amplification product was sent to Macrogen (Singapore) 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 nucleotide sequences obtained were then analyzed using the multiple alignment ClustalW in Bioedit sequence alignment editor software version 7.0.5. The homology results are close to 100% similarity categorized as the same species as the sample species.

RESULTS AND DISSCUSSION

Disease Studies

Bitter rot disease (anthracnose) of apples shows symptoms of brown to black rot (Figure 1B) very much different from healthy apples (Figure 1A). The white mushroom mycelium gradually turned black (Figure 1C). The results of observing the pathogen under the microscope turned out that the conidia appeared small round (Fig. 1D).

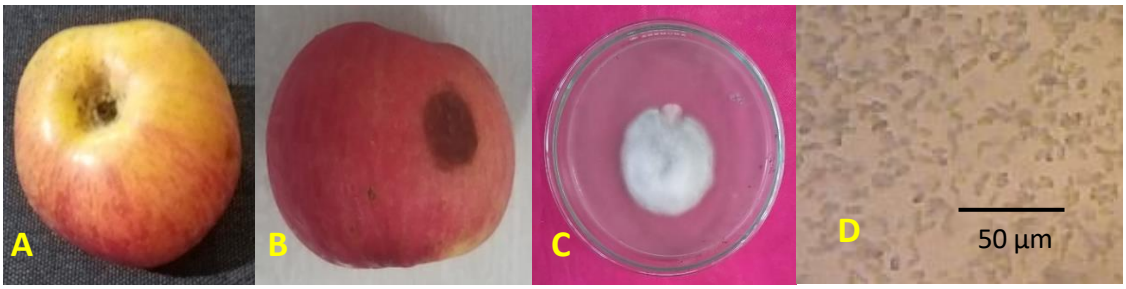

Figure 3. Observations of disease in apples. (A) healthy fruit, (B) diseased fruit, (C) mycelium colonies in Petri dish, and (D) conidia at 100x magnification

Bitter rot of apples caused by *Colletotrichum* species is a growing problem worldwide. *Colletotrichum* sp. economically important (Khodadadi *et al*., 2020). *Colletotrichum* spp. can rest on hardened fruit, infected vegetative tissue or asymptomatic plants, eg. grapes, while *C. gloeosporioides*was found to overseason on hardened fruit, and pedicles. In apples both the sexual and asexual stages of *C. gloeosporioides* rest on dead wood and perithesia develops on infected fallen leaves. During the hot rainy season, ascospores and conidia are released initiating primary infection on growing leaves and fruit (Dowling *et al*., 2020).

Bitter rot caused by a fungus of the genus *Colletotrichum* is one of the most common apple fruit diseases and causes significant yield loss in apple trees. *C. gloeosporioides* and *C. acutatum* are the two most common species that cause bitter rot in apples. *Collectitrichum* is difficult to identify species based solely on morphology, due to the similarity of morphology under different growing conditions. Recently, *Colletotrichum* was studied based on morphological and multilocus phylogenetic analysis, grouping 189 recognized species into 11 species complexes. Additional studies described several new species from different hosts in the *C. acutatum* and *C. gloeosporioides* complexes (Park *et al.*, 2018).

Molecular Identification

DNA fragments of 650 bp in size were successfully amplified from 6 samples of fungi using the universal primer ITS1/ITS4 (Figure 2). The amplified DNA sample is then used for the sequencing stage to determine the species of the fungus. Sequential analysis confirmed that the identity of the fungus in the sample was *C.gloeosporioides* with 78-100% homology respectively to several *C. gloeosporioides* isolates and out group in genebank (Table 1).

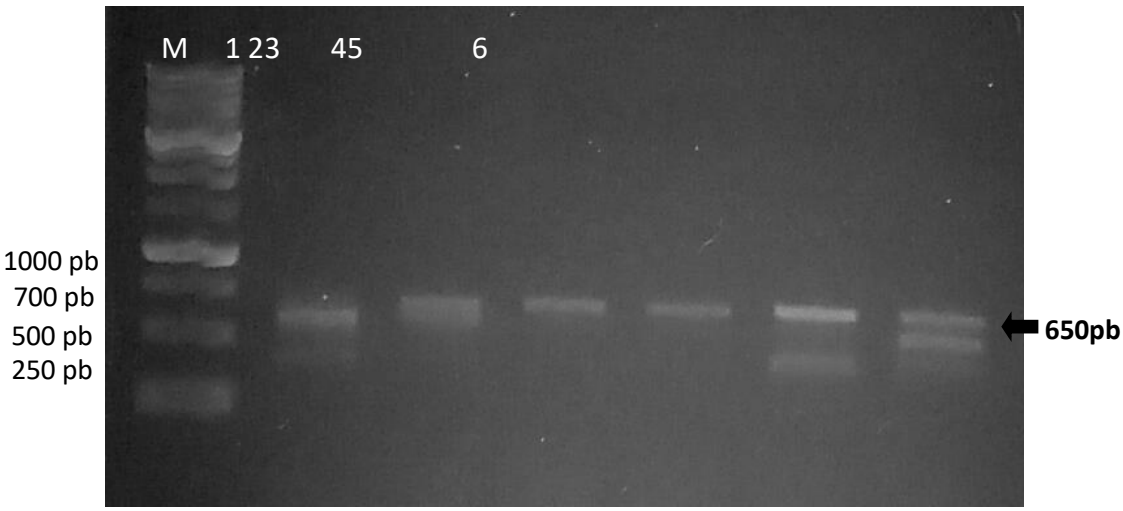

Figure 2. Visualization of *Colletotrichum gloeosporioides* DNA amplified using universal primer ITS1/ITS4 on 1% agarose gel. M: DNA marker (1kb ladder); Sample no. 1-6 (Balinese Isolate)

Bitter rot of apples caused by *C.gloeosporioides* was first reported in China in 1985. In China, apples are grown on approximately 2 million ha, and bitter rot occurs in almost all production areas, with crop damage ranging from 30 to 70%. During the summer of 2007, the fungus was isolated from apples showing bitter rot symptoms in 12 and 9 orchards in Shaanxi and Henan provinces, respectively in China. Symptoms include 2 to 3 cm diameter, sunken, brown lesions on the surface of the fruit that contain black, pinhead-sized fruit structures that produce masses of orange conidia under high humidity, similar to those of *C. gloeosporioides*. On potato dextrose agar (PDA), colonies are white, pale gray, or pale orange when grown at 25°C. Conidia 8 to 16 × 2.5 to 4 µm, fusiform, tapered at one or both ends, single-celled, thin-walled, septate, and hyaline. Appresoria measures 6.5 to 11 × 4.5 to 7.5 µm, clavate to circular, and light to dark brown (Zhang *et al*., 2008).

Colletotrichum spp. present in tropical and subtropical regions around the world. *Colletotrichum gloeosporioides* is the most important pathogen and belongs to the Melanconial order. These pathogens establish interactions with the host by producing an appresorium, undergo melanization and then penetrate the host

cuticle. After penetration, infecting vesicles and primary hyphae are formed, then secondary hyphae develop and spread to kill host cells. *Colletotrichum gloeosporioides* follows a hemibiotrophic mode of infection in which the biotrophic and necrotrophic phases occur sequentially (Sharma and Kulshrestha, 2015).

Table 1. Homology (%) of the nucleotide sequences of *Colletotrichum gloeosporioides* isolates with several isolates that have been reported in GenBank

Sequence	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	G (%)	H (%)	I (%)	J (%)	H (%)	I (%)
A	ID	100	100	100	100	100	100	100	100	100	100	78
B	100	ID	100	100	100	100	100	100	100	100	100	78
C	100	100	ID	100	100	100	100	100	100	100	100	78
D	100	100	100	ID	100	100	100	100	100	100	100	78
E	100	100	100	100	ID	100	100	100	100	100	100	78
F	100	100	100	100	100	ID	100	100	100	100	100	78
G	100	100	100	100	100	100	ID	100	100	100	100	78
H	100	100	100	100	100	100	100	ID	100	100	100	78
I	10	100	100	10	100	100	100	100	ID	10	100	78
J	100	100	100	100	100	100	100	100	100	ID	100	78
K	100	100	100	100	100	100	100	100	100	100	ID	78
L	78	78	78	78	78	78	78	78	78	78	78	ID

Where: A = *C. gloeosporioides*, Bali. B = MK530178 *C. gloeosporioides* CHI, C = MN075768 *C. gloeosporioides* CHI, D = MK644101 *C. gloeosporioides* CHI, E = MK790665 *C. gloeosporioides* PRO, F = MH520670 *C. gloeosporioides* PAK, G = LC406907 *C. gloeosporioides* KOR., H = MH571757 *C. gloeosporioides*PAK., I = KX578796 *C. gloeosporioides* BRA, J = MZ822130 *C. gloeosporioides* CHI., H = MZ312234 *C. gloeosporioides* CHI., dan I = MH879859 *Fusarium oxysporum* PAK.

Bitter rot disease also attacks Brazilian apples. Apple cultivars are susceptible to several diseases that can cause losses after harvest. Bitter rot is caused by the fungus *C. gloeosporioides* and is one of the most destructive summer diseases. The study was conducted on four apple cultivars grown in Brazil (Fuji, Gala, Golden and Green) under two treatments: direct inoculation and isolated fungus. Isolation of the fungus was carried out by taking infected fruit pieces stored on Potato Dextrose Agar (PDA) in the laboratory. For direct treatment, the fungus is removed from infected fruit and immediately inoculated into healthy fruit. After inoculation, the fruit was stored at room temperature and halo degradation was evaluated every 48 hours. The results showed that direct inoculation was more pathogenic, that the Gala cultivar was the most resistant to pathogens, and that the Gold cultivar was the most susceptible (Onofre and Antoniazzi, 2014).

Further phylogenetic analysis showed that *C. gloeosporioides* isolates formed three groups. Isolate *C_gloeosporioides*_Bali formed a group with 7 isolates from Asia from the genebank including isolates from China, South Korea and Pakistan. The second group consists of Pakistan and Brazil, while the third group forms a separate group with other isolates. As an out group isolate, *Fusarium oxysporum*isloate from Pakistan was used (Figure 3).

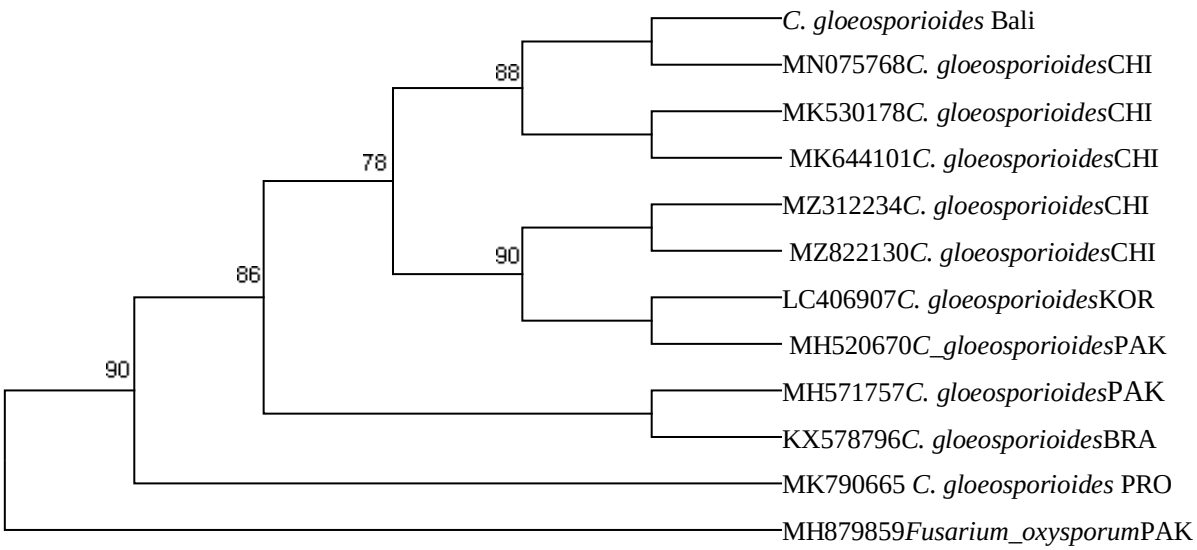

Figure 3. Phylogenetic analysis of the isolate *Colletotrichum gloeosporioides* based on partial nucleotide sequence alignment of the internally transcribed spacer 1, 5.8S ribosomal RNA gene using Mega 6.06 (Neighbor Joining Algorithm with 1,000 bootstraps replicates)

CONCLUSION

Bitter rot disease (anthracnose) in apples is caused by *Colletotrichum gloeosporioides* with a 650 bp DNA fragment. After matching with genbank, 100% homology percentage can be concluded that anthracnose disease in apples is caused by *C. gloeosporioides*.

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