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Morphological, molecular, and pathogenic characterization of *Colletotrichum gloeosporioides* sensu stricto associated with imported citrus fruits

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Abstract. *Colletotrichum gloeosporioides* is a fungal pathogen that causes anthracnose disease in citrus fruits. *C. gloeosporioides* has the potential to be carried by imported citrus fruits. The aim of this study was to detect, isolate, identify, and test the pathogenicity of *C. gloeosporioides* associated with imported citrus fruits. The detection method was carried out by freezing imported citrus fruits at -20 °C for 15 hours. *C. gloeosporioides* was identified morphologically and molecularly. The morphological identification method was done by observing mycelial colonies and conidia. The molecular identification method was done by the Polymerase Chain Reaction technique using 3 locus of ITS, GAPDH, and TUB2. The pathogenicity was tested by attaching 1 culture circle of *C. gloeosporioides* to the surface of the citrus fruit and injecting 100 µl of conidia suspension at a density of 10^5 conidia mL^{-1} into the inside of the citrus fruit. The main results showed that *C. gloeosporioides* was successfully detected at 4 days after incubation by the emergence of mycelia on the surface of citrus fruits. Morphological and molecular identification proved that the species of fungus was *C. gloeosporioides* sensu stricto which was a pathogenic fungus on imported and local citrus fruits with a disease incidence value of 100%, and a disease severity value of 97.78% and 98.89%, respectively.

Key words: anthracnose, disease incidence, disease severity, identification, pathogenicity.

INTRODUCTION

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. is a pathogenic fungus that causes anthracnose disease in citrus fruits (Patricia et al., 2021). Anthracnose is an important disease on citrus fruits because it can cause economic losses from the field to storage. Yield losses caused by *C. gloeosporioides* infection can reach 80% (Silva-Junior et al., 2014). Therefore, Dean et al. (2012) categorized *C. gloeosporioides* into the 10 most important of plant pathogenic fungi in the world. Symptoms of anthracnose include light brown to black necrotic lesions on almost the entire

surface and blackish brown rot on the inside of citrus fruits (Cruz-Lagunas et al., 2020; Khamsaw et al., 2022).

Anthracnose disease is spread in almost all citrus plantations in the world (Perez-Mora et al., 2020). *C. gloeosporioides* was also reported to attack citrus plantations in several areas in Indonesia. Suryaningsih et al. (2015) isolated *C. gloeosporioides* from citrus fruits with anthracnose symptoms in citrus plantations in Bali. The most recent report was submitted by Wiyanna et al. (2022) who have characterized *C. gloeosporioides* from citrus plantations in Singkawang, West Kalimantan. The geographic distribution of plant pathogens influences the level of pathogen virulence (Shaw & Osborne, 2011). This means that *C. gloeosporioides* species from various countries are thought to have different characters and levels of virulence. Sacristan & Garcia-Arenal (2008) stated that the differences in character were caused by differences in pathogen strains.

Indonesia is still importing citrus fruits from various countries, such as Pakistan, Australia, China, and Argentina. Lichtenberg & Olson (2018) stated that imported citrus fruits have the potential to carry pathogens that cause plant diseases. This potential is based on the ability of the pathogen to latently infect citrus fruits without expressing disease symptoms on the surface of the fruit (Johnston et al., 2005). Infected fruits with a latent fungus look as healthy and normal as uninfected fruits. Latent infectious fungi were difficult to be detected and isolated (Schaad et al., 2003). This detection difficulty raises concerns that latent infectious fungi on imported citrus fruits will be brought and spread to local citrus fruit plantations. *C. gloeosporioides* is a fungus that is known to be able to latently infect postharvest fruits (Agrios, 2005). *C. gloeosporioides* infects latently by forming an inactive structure in the fruit when the physical condition of the fruit is not yet possible to be infected (Prusky & Lichter, 2007). Droby & Wisniewski (2018) explained that the fungus starts the infection when the postharvest fruits have undergone a senescence process. Michailides et al. (2010) added that the physicality of fruits is weakened during the senescence process.

C. gloeosporioides infects latently in citrus fruits and being difficult to be detected. Latent infection in fruits can be physically detected. Michailides et al. (2010) reported that *Monilinia fructicola* and *M. laxa* causing brown rot in fruits, *Botrytis cinerea* causing rot in grapes and *Alternaria* causing blight in nuts, can be physically detected within 5 to 7 days using the Overnight Freezing Incubation Technique (ONFIT). *C. gloeosporioides* is also difficult to be identified accurately. According to Cai et al. (2009), the difficulty in identifying *C. gloeosporioides* is caused by the number of morphological characters as biological markers are few and varied, and has a wide host range with varying levels of pathogenicity. Therefore, identification of *C. gloeosporioides* through morphological characters is not enough, but needs further identification through a polyphasic approach by combining identification using genetic and physiological characters. Species of a pathogen is essential to be identified to understand the epidemiology and to develop effective controls. Therefore, even though *C. gloeosporioides* is already an endemic fungal pathogen in Indonesia, but its presence in imported citrus fruits must still be characterized because it comes from a different geographical area. Moendeg et al. (2017) explained the difference in geographical areas can cause different strains of pathogens.

This study aims to detect, isolate, identify, and test the pathogenicity of *C. gloeosporioides* carried by imported citrus fruits. The research results are expected to be used as guidance for consideration in mitigating the risk of the spread of *C. gloeosporioides*.

MATERIALS AND METHODS

Research Locations, time, and citrus fruits

The research was conducted at the Plant Mycology Laboratory, Department of Plant Protection, IPB University, Bogor Regency and Biotechnology Laboratory, Applied Research Institute of Agricultural Quarantine, Bekasi Regency from December 2021 to October 2022. Citrus fruits come from imported commodities obtained from Tanjung Priok Port, Jakarta, Indonesia. The variety of citrus fruit is mandarin (*Citrus reticulata*). Citrus fruits used in the study met the criteria of being healthy without showing disease symptoms and were uniform in shape, size, and color.

Detection and isolation of *C. gloeosporioides*

C. gloeosporioides was detected using the Overnight Freezing Incubation Technique (ONFIT). Citrus fruits were washed using tap water to clean all material attached to the surface of the fruit and rinsed 2 times using sterile water. Citrus fruits were dried and placed on a rack in a 35×27×10 cm plastic container (Fig. 1). Sterile water was added to the plastic container to maintain moisture. The plastic container was tightly closed and stored in the freezer at -20 °C for 15 hours. Citrus fruits in plastic containers were incubated at room temperature. The mycelia of *C. gloeosporioides* that appeared on the surface of citrus fruits were isolated and grown on PDA media.

Identification of *C. gloeosporioides* morphologically and molecularly was carried out using pure cultures to ensure that the identification process was conducted only on 1 individual species not mixed with other species (contaminants). Pure cultures were prepared by the conidial serial dilution technique. A total of 5 mycelial circles of *C. gloeosporioides* culture on PDA media were taken using a cork borer (4.5 mm in diameter). The culture circle was put into 10 mL of sterile distilled water and shaken. A total of 1 mL of sterile distilled water mixed with mycelia and conidia of *C. gloeosporioides* was taken and transferred to 9 mL of sterile distilled water (10^{-1}). Dilution was continued until the mixture between the mycelia and the conidia of *C. gloeosporioides* did not look thick. A total of 0.1 mL of each dilution was taken and spread on PDA media. The mycelia that grew from 1 conidium were removed, transferred, and re-grown onto new PDA media as a pure culture.

Morphological identification of *C. gloeosporioides*

C. gloeosporioides was identified morphologically from a pure culture based on the characteristics of the fungi's identifying organs, such as mycelia and conidia. Identification of mycelia was carried out by observing the growth pattern of mycelial colonies on PDA media which included texture and color. Conidia were identified morphometrically by observing the shape and measuring 50 conidia randomly. The decision of morphological identification is based on the similarity between the obtained morphological character data and the identification key from several available references.

DNA extraction of *C. gloeosporioides*

The DNA of *C. gloeosporioides* was extracted using 2 conventional methods by Abd-Elsalam et al. (2003) with some modifications and Ausubel et al. (2003) with some modifications. The pure culture of the *C. gloeosporioides* used in the DNA extraction process was 7 days old.

1) Abd-Elsalam et al. (2003) with some modifications.

The mycelia of the *C. gloeosporioides* were taken from the pure culture and inoculated into 500 µl of Potato Dextrose Broth (PDB) medium in a 1.5 mL tube. Fungal mycelia in PDB was shaken at 150 rpm at room temperature for 72 hours. The growing mycelia were pelleted using a centrifugation technique at 13,000 rpm for 5 minutes at room temperature. The liquid phase (supernatant) was discarded and the pellet was washed with 500 µl of Tris-EDTA solution (pH 8.0). The pellet was again centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and the pellet was crushed using a pistil. 300 µl of extraction solution (200 mm Tris-HCl [pH 8.5], 250 mm NaCl, 25 mm EDTA, and 0.5% sodium dodecyl sulfate) was added to the pellet and homogenized with a pistil for 5 minutes. 150 µl of 3M sodium acetate (pH 5.2) was added and cooled at -20 °C for 10 minutes. The pellet was centrifuged at 13,000 rpm for 5 minutes. The resulting liquid (supernatant) was measured and transferred to a new 1.5 mL tube. An equal volume of isopropanol with supernatant was added and centrifuged at 13,000 rpm for 5 minutes. DNA was washed by adding 500 µl 70% ethanol and centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the DNA was dried. DNA was suspended in 100 µl Tris-EDTA solution (10 mm Tris-HCl [pH 8.0], 1 mm EDTA). The DNA suspension was stored at -20 °C.

2) Ausubel et al. (2003) with some modifications.

A total of 10 mycelial circles from a pure culture of *C. gloeosporioides* were taken using a 4.5 mm diameter of cork borer. The mycelial circles were put into a sterile aluminum foil container with a tight cover and stored in the freezer -80 °C for 3 hours. The frozen mycelial circles were crushed until smooth using a mortar and dissolved by adding 600 µl of a mixed solution of CTAB extraction and 2% mercaptoethanol which had been previously heated in a water bath at 65 °C. The mycelial suspension was put into a 1.5 mL tube and heated in a water bath at 65 °C for 60 minutes with occasional homogenization by hand. 600 µl CI (24:1) was added, homogenized, and centrifuged at 10,000 rpm at 4°C for 5 minutes. The supernatant was taken, measured, and transferred to a new 1.5 mL tube. The CTAB/NaCl solution that had been preheated in a 65 °C water bath was taken as much as 1 10^{-1} of the volume of the supernatant and stirred until evenly distributed. CI (24:1) with the same volume was added, shaken, and centrifuged at 10,000 rpm at 4 °C for 5 minutes. The supernatant was taken, measured, and transferred to a new 1.5 mL tube. CTAB precipitation solution of as much as 1 volume of supernatant was added, stirred evenly, and centrifuged at 2,700 rpm at 4 °C for 5 minutes. 150 µl of TE solution with high salt content was added and shaken evenly. DNA was precipitated by adding isopropanol as much as 0.6 volume of the total solution and centrifuged at 10,000 rpm at 4 °C for 20 minutes. The supernatant was discarded, and the DNA pellet was washed by adding 500 µl 80% ethanol and centrifuged at 8,000 rpm for 5 minutes at room temperature. The remaining ethanol was discarded and the DNA pellet was dried. DNA was suspended by adding 100 µl of TE solution. The DNA suspension was stored at -20 °C.

The concentration of extracted *C. gloeosporioides* DNA was measured using Nanodrop One with serial number AZY2125665. DNA measurement aims to assess the quantity of obtained DNA from the two extraction methods used.

Molecular identification of *C. gloeosporioides*

ITS, GAPDH, and TUB2 genes were amplified using primers pair of ITS1 and ITS4, GDF1 and GDR1, Btub2Fd and Btub4Rd through PCR technique. Amplicon nucleotide bases were sequenced. The DNA sequence was identified using the Basic Local Alignment Search Tool program (BLAST) on the website https://www.ncbi.nlm.nih.gov/.

DNA Amplification of the ITS, GAPDH, and TUB2 locus of *C. gloeosporioides*

The *C. gloeosporioides* DNA was amplified using primer pairs of ITS with an amplicon size of around 600 bp, GAPDH with an amplicon size of around 200 bp, and TUB2 with an amplicon size of around 600 bp. The sequences of all primers were shown in Table 1. The amplification process was carried out by PCR technique using the 'Applied Biosystems type Veriti' thermal cycler. The PCR reaction was conducted in a 50 µl volume containing 20 µl PCR master mix, 2 µl ITS1F primer, 2 µl ITS4 primer, 4 µl extracted DNA, and 22 µl ddH2O. ITS and GAPDH amplification was carried out for 35 cycles with the steps of DNA double-strand denaturation at 94 °C for 35 seconds, primer attachment to the ITS DNA locus at 51 °C for 1 minute, and DNA elongation at 72 °C for 2 minutes (White et al., 1990; Gardes & Bruns, 1993; Guerber et al., 2003). TUB2 amplification was carried out for 35 cycles with the steps of DNA double-strand denaturation at 94 °C for 30 seconds, primer attachment to the ITS DNA locus at 52 °C for 30 seconds, and DNA elongation at 72 °C for 30 seconds (Woudenberg et al., 2009). The results of DNA amplification were visualized on 1% agarose gel.

Table 1. List of primers used in this study

Locus	Primer name	Sequence (5'-to-3')	References
ITS	ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
	ITS4	CCTCCGCTTATTGATATGC	White et al. (1990)
GAPDH	GDF1	GCCGTCAACGACCCCTTCATTGA	Guerber et al. (2003)
	GDR1	GGGTGGAGTCGTACTTGAGCATGT	Guerber et al. (2003)
TUB2	Btub2Fd	GTBCACCTYCARACCGGYCARTG	Woudenberg et al. (2009)
	Btub4Rd	CCRGAYTGRCCRAARACRAAGTTGTC	Woudenberg et al. (2009)

The ITS, GAPDH, and TUB2 locus sequencing of the *C. gloeosporioides* DNA

The PCR product containing the ITS, GAPDH, and TUB2 amplicon of *C. gloeosporioides* DNA was sequenced at First Base, Malaysia. The DNA nucleotide sequences of *C. gloeosporioides* were analyzed by BLAST on the NCBI website. The BLAST process was carried out to identify DNA sequences based on species that have the highest DNA sequence similarities in NCBI GenBank.

Phylogenetic tree

The genetic relationship between *C. gloeosporioides* from local and imported citrus fruits, and other countries was determined by constructing a phylogenetic tree. The phylogenetic tree was built using Mega software version 10, through a neighbor-joining approach with a bootstrap value of 1,000 times.

Pathogenicity Test

Pathogenicity test of *C. gloeosporioides* used imported and local healthy mandarin citrus fruits without showing disease symptoms. The pathogenicity test was carried out using the attachment technique of *C. gloeosporioides* culture to the surface of citrus fruits and the injection technique of *C. gloeosporioides* conidia suspension into citrus fruits. Citrus fruits were surface sterilized before being used. Citrus fruits were immersed in a 5.26% sodium hypochlorite solution for 4 minutes to disinfect surface pathogens. Citrus fruits were rinsed 2 times using sterile water and dried on sterile tissue paper. Ten citrus fruits were arranged on a rack in a sterile plastic container of 35×27×10 cm and tightly closed. Sterile water was added to the plastic container to make suitable humidity for *C. gloeosporioides* growth.

1) The attachment technique of *C. gloeosporioides* culture to the surface of citrus fruits.

The *C. gloeosporioides* culture was taken using a 4.5 mm of cork borer. One side of the upper surface of the citrus fruit was injured using a sterile syringe from the outer surface to the deepest skin. One circle of culture was attached to the surface of the citrus fruit that has been injured. Injury treatment and culture attachment were carried out on 10 citrus fruits which had been arranged on a rack in a plastic container. The control treatment was wounded by a sterile syringe and followed by the attachment of 1 circle of blank PDA media without *C. gloeosporioides* culture to 10 citrus fruits. Citrus fruits were incubated at room temperature for 21 days. The treatment of culture attachment and control was conducted with 3 replications.

2) The injection technique of *C. gloeosporioides* conidia suspension into citrus fruits.

Ten citrus fruits in a plastic container were injected using a sterile syringe containing 100 µl of *C. gloeosporioides* conidia suspension at a density of 10^5 conidia mL^{-1} into the columella of the citrus fruit through the base of the calyx. Citrus fruits were injected with 100 µl of sterile water in the control treatment. Citrus fruits were incubated at room temperature for 21 days. The injection treatment of conidial suspension and control was repeated 3 times (Camiletii et al., 2022).

Citrus fruits were observed by assessing the broad scale of anthracnose symptoms caused by *C. gloeosporioides*. Symptoms were observed from the outside and inside of citrus fruits. Observation of internal symptoms was carried out by splitting citrus fruits. Scale 0 = no visible symptoms; 1 = 1–30% symptom; 2 = 30–60% symptom; and 3 = ≥ 60% symptoms (Mojerlou & Safaie, 2012). The pathogenicity of *C. gloeosporioides* was determined by assessing the incidence and severity of the disease based on the appearance and scale of anthracnose symptoms on citrus fruits. The incidence and severity of the disease were calculated using the following formula:

$$DI = \frac{\text{Number of rotten fruit}}{\text{Total number of fruits observed}} \text{ x } 100\%$$

$$DS = \sum \frac{(n \text{ x } V)}{Z \text{ x } N} \text{ x } 100\%$$

where DI – Disease Incidence; DS – Disease Severity; n – number of fruits showing the same scale; V – symptom scale; Z – the highest scale; N – total number of fruits observed.

Pathogenicity test used a Completely Randomized Design (CRD). Data were analyzed by Analysis of Variance (ANOVA). If the results of the analysis show a *P*-value < α = 0.05, then proceed with the Tukey test at level α = 0.05 to determine differences in disease severity between the pathogenicity treatments of

C. gloeosporioides and control (Kasiamdari & Sangadah, 2015). Analysis was performed using SAS software version 9.4.

RESULTS AND DISCUSSION

Results

Detection and isolation of *C. gloeosporioides*

C. gloeosporioides was successfully detected at 4 Days After Incubation (DAI) using the ONFIT method through the emergence of mycelia on the surface of imported citrus fruits. Fig. 1 shows grayish-white mycelia on the surface of citrus fruits, like coarse cotton with a flat and spreading growth pattern. The ONFIT treatment caused skin discoloration of the citrus fruit from orange to dark brown and the skin texture became dry. Meanwhile, citrus fruits in the control treatment still look fresh and orange in color (Fig. 1). Table 2 shows 37% of the imported citrus fruits carried *C. gloeosporioides*. Citrus fruits were not covered by mycelia at all in the control treatment without the ONFIT method.

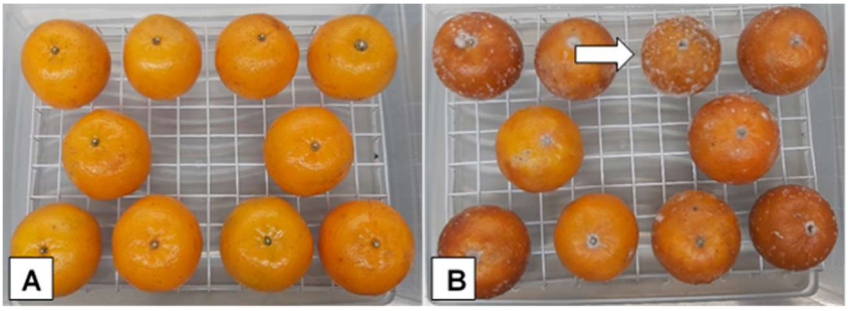

Figure 1. The emergence of *C. gloeosporioides* mycelia on the surface of imported citrus fruits at 4 DAI. A – Control treatment; B – ONFIT treatment.

Table 2. The emergence percentage of *C. gloeosporioides* mycelia on the surface of imported citrus fruits at 4 DAI

Treatment	Characterization of mycelia on the surface of citrus fruits	Amount	Percentage of emergence (%)[1]
Control	No mycelia found	0	0
ONFIT	Mycelial colonies are grayish-white, like coarse cotton, and grow flat	37	37

[1] Percentage of emergence was obtained from the number of mycelial emergences in 100 citrus fruits. The emergence of the same mycelia in more than one of the same citrus fruit was counted as 1.

Morphological identification of *C. gloeosporioides*

Colonies of *C. gloeosporioides* mycelia that grew from a single conidium on PDA media had a diameter of 6.74–7.01 (mean 6.82, $n = 5$) cm at 7 DAI (Table 3). Characteristics of mycelia were aerial types, such as coarse cotton, and grayish-white. Conidia size were 8.20–21.20×3.80–5.50 (mean 15.28×4.59, $n = 50$) µm, hyaline, cylindrical, non-septate, broadly rounded at one end, and tapered at the other (Fig. 2).

Table 3. Morphological characterization of *C. gloeosporioides* from imported citrus fruits

Morphological characterization	Description
Macroscopic characteristics	
Colonies on the surface of citrus fruits[1]	
Color	Grayish-white
Texture of mycelia	Like coarse cotton
Colonies on PDA media[2]	
Color	Grayish-white
Form	Circular
Elevation	Flat
Margin	Undulate
Pigmentation[3]	No pigmentation
Growth rate (cm)[4]	6.82 ± 0.13
Microscopic characteristics	
Conidia morphology	Cylindrical and slender
Apical cell morphology	Broadly rounded
Basal cell morphology	Tapered
Mean length of conidia (µm)[5]	15.28 ± 2.08
Mean width of conidia (µm)[6]	4.59 ± 0.40
Septation	Non-septate

[1]Colonies were observed at the surface of the citrus fruits; [2]Colonies were observed at the top of the culture of *C. gloeosporioides*; [3]Pigmentation was observed on the bottom of the culture of *C. gloeosporioides*; [4]The mycelial growth rate of *C. gloeosporioides* was observed at 7 DAI; [5,6]The mean length and width of conidia, were observed from 50 randomly selected conidia ± standard deviation.

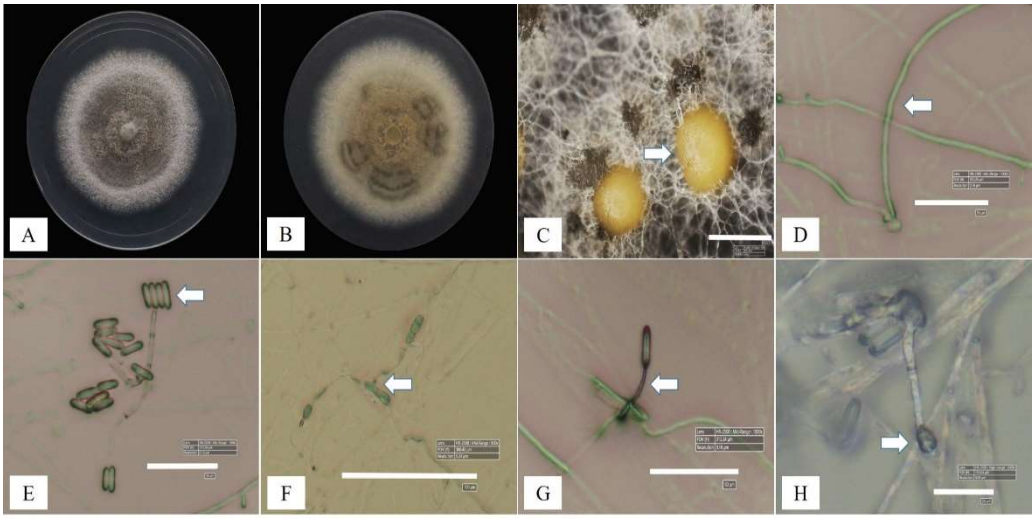

Figure 2. Morphological characteristics of *C. gloeosporioides* from imported citrus fruits. A – Fungal colonies 7 days old on upper PDA media; B – Bottom part of fungal culture; C – Conidiomata on PDA, scale bar = 500 µm; D – Hyphae, scale bar = 50 µm; E – Conidia, scale bar = 50 µm; F – Hyphae formed from a conidium, scale bar = 100 µm; G – Conidiophore, scale bar = 50 µm; H – Appressorium developed from a conidium with a long germ tube, scale bar = 20 µm.

DNA extraction of *C. gloeosporioides*

Both DNA extraction methods succeeded in obtaining the DNA of *C. gloeosporioides*. However, DNA measurements using nanodrop of the two extraction methods yielded different concentrations. Table 4 informs that the DNA extraction method from Abd-Elsalam et al. (2003) produced the highest DNA concentration of 487.806 ng μl^{-1} compared to the Ausubel et al. (2003) method of 56.868 ng μl^{-1}. Abd-Elsalam et al. (2003) method also had a higher A260/A280 value of 2.175 compared to the Ausubel et al. (2003) method of 1.620. However, the extraction time of the Abd-Elsalam et al. (2003) method was about 3 days and 2 hours longer than the Ausubel et al. (2003) method about 6 hours.

Table 4. DNA quantification using nanodrop

DNA extraction methods	Concentration (ng μl^{-1})	A260/A280	Extraction time
Abd-Elsalam et al. (2003)	487.806 ± 35.350	2.175 ± 0.016	± 3 days and 2 hours
Ausubel et al. (2003)	56.868 ± 8.307	1.620 ± 0.014	± 6 hours

Molecular identification of *C. gloeosporioides*

DNA Amplification of the *C. gloeosporioides* ITS, GAPDH, and TUB2 locus

The extracted DNA from both methods was successfully amplified by ITS1 and ITS4 primers at the ITS locus, GDR1 and GDF1 at the GAPDH locus, Btub2Fd and Btub4Rd at the TUB2 locus. Visualization of DNA bands on 1% agarose gel can be seen in both extraction methods according to the ITS target size of ± 600 bp. Fig. 3 shows the total DNA concentration correlated with the visualization of the DNA bands. Abd-Elsalam et al. (2003) method which has the highest DNA concentration shows the DNA bands thicker than the Ausubel et al. (2003).

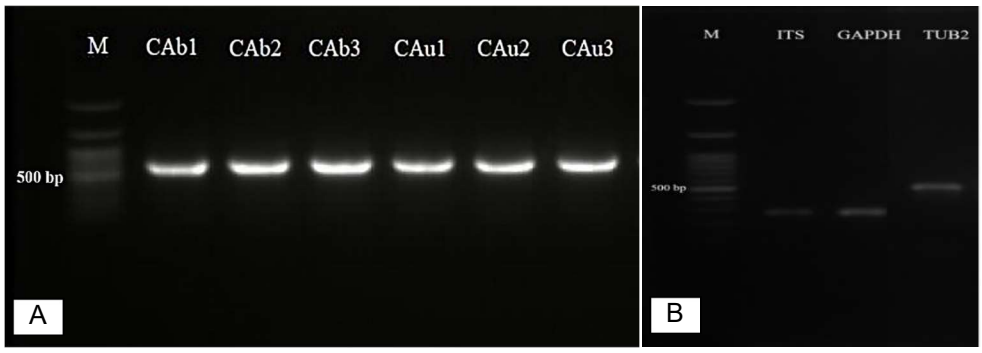

Figure 3. Visualization of DNA bands. A – DNA bands from all DNA extraction methods using universal primer pairs ITS1 and ITS4. CAb1–CAb3 are Abd-Elsalam et al. (2003) method with 3 replications and CAu1–CAu3 are Ausubel et al. (2003) method with 3 replications. B – DNA bands from locus ITS, GAPDH, and TUB2.

DNA sequencing of the *C. gloeosporioides* ITS, GAPDH, TUB2 locus

DNA amplicon that has been sequenced was subjected to the BLAST process on the NCBI website. BLAST results showed that the ITS locus amplicon of *C. gloeosporioides* DNA from imported citrus fruits had the highest DNA sequence similarity with isolate *Colletotrichum gloeosporioides* COL5 from Pakistan with

accession number MH645072.1. The GAPDH locus amplicon of *C. gloeosporioides* DNA had the highest DNA sequence similarity with isolate *Colletotrichum gloeosporioides* WT-Y4 from China with accession number MK940674.1. The TUB2 locus amplicon of *C. gloeosporioides* DNA had the highest DNA sequence similarity with isolate *Colletotrichum gloeosporioides* strain 8 from Italy with accession number JN121296.1. Table 5 presents the top 5 of BLAST results of *C. gloeosporioides* from imported citrus fruits with various isolates showing the highest similarity.

Table 5. The similarity of *C. gloeosporioides* isolate from imported citrus fruit with other isolates in GenBank

Isolate identity from imported citrus fruits	Locus	Isolate identity in *GenBank*	Accession number	Query cover (%)	Similarity (%)	Origin
Colletotrichum gloeosporioides isolate CGJIPKN1	ITS	*Colletotrichum gloeosporioides* COL5	MH645072.1	100	98.55	Pakistan
		Colletotrichum gloeosporioides	MH520670.1	100	98.39	Pakistan
		Colletotrichum gloeosporioides strain AGMy0229a	KX578796.1	100	98.07	Brazil
		Colletotrichum siamense SY71	OM967145.1	100	97.91	China
		Colletotrichum fructicola C09320	MW368667.1	100	97.59	South Korea
	GAPDH	*Colletotrichum gloeosporioides* WT-Y4	MK940674.1	100	100	China
		Colletotrichum gloeosporioides	MH321225.1	100	100	China
		Colletotrichum gloeosporioides strain AGMy0075	KX578776.1	100	100	South Africa
		Colletotrichum gloeosporioides	KU612891.1	100	100	Australia
		Colletotrichum gloeosporioides	ON050981.1	100	100	Colombia
	TUB2	*Colletotrichum gloeosporioides* Strain 8	JN121296.1	99	99.62	Italy
		Colletotrichum gloeosporioides	MT409132.1	98	99.62	Italy
		Colletotrichum gloeosporioides Strain HNHJ-73	MN908602.1	98	99.62	China
		Colletotrichum gloeosporioides Strain FMB 0137	MN339477.1	98	99.62	Pakistan
		Colletotrichum gloeosporioides Strain BRIP 66213	MK390653.1	98	99.62	Australia

Phylogenetic tree

Several strains of the *C. gloeosporioides* species complex listed in Table 6 have been determined for their genetic relationship through a phylogenetic tree. Fig. 4 shows that *C. gloeosporioides* from imported citrus fruits has a close genetic relationship with *C. gloeosporioides* isolates from Italy, Australia, Banyuwangi and Malang, Indonesia. These findings prove that *C. gloeosporioides* from imported citrus fruits is the same species as *C. gloeosporioides* from Banyuwangi and Malang, Indonesia.

Table 6. A list of *C. gloeosporioides* species complex strains used in this study

Species	Host	Country	GenBank Accession Number		
			ITS	GAPDH	TUB2
C. aenigma	*Persea americana*	Israel	JX010244	JX010044	JX010389
	Pyrus pyrifolia	Japan	JX010243	JX009913	JX010390
C. aeschynomenes	Aeschynomene virginica	USA	JX010176	JX009930	JX010392
C. alatae	*Dioscorea alata*	India	JX010190	JX009990	JX010383
	Dioscorea alata	Nigeria	JX010191	JX010011	JX010449
C. alienum	*Persea americana*	Australia	JX010217	JX010018	JX010385
	Malus domestica	New Zealand	JX010251	JX010028	JX010411
C. aotearoa	*Vitex lucens*	New Zealand	JX010220	JX009906	JX010421
	Kunzea ericoides	New Zealand	JX010198	JX009991	JX010418
C. asianum	*Mangifera indica*	Australia	JX010192	JX009915	JX010384
	Coffea arabica	Thailand	FJ972612	JX010053	JX010406
C. clidemiae	*Vitis sp.*	USA	JX010274	JX009909	JX010439
	Clidemia hirta	USA	JX010265	JX009989	JX010438
C. cordylinicola	*Cordyline fruticosa*	Thailand	JX010226	JX009975	JX010440
C. fructicola	*Coffea arabica*	Thailand	JX010165	JX010033	JX010405
	Fragaria x ananassa	USA	JX010179	JX010035	JX010394
C. gloeosporioides	*Citrus sinensis*	Italy	JX010152	JX010056	JX010445
	Citrus reticulata	Australia	MG572144	MG572133	MG572155
C. horii	*Diospyros kaki*	New Zealand	GQ329687	GQ329685	JX010375
	Diospyros kaki	China	JX010212	GQ329682	JX010378
C. kahawae subsp. ciggaro	*Olea europaea*	Australia	JX010230	JX009966	JX010434
	Kunzea ericoides	New Zealand	JX010227	JX009904	JX010427
C. kahawae subsp. kahawae	*Coffea arabica*	Angola	JX010234	JX010040	JX010435
	Coffea arabica	Cameroon	JX010232	JX010046	JX010431
C. musae	*Musa sapientum*	Kenya	JX010142	JX010015	JX010395
	Musa sp.	USA	JX010146	JX010050	HQ596280
C. nupharicola	*Nuphar lutea subsp. polysepala*	USA	JX010189	JX009936	JX010397
C. psidii	*Psidium sp.*	Italy	JX010219	JX009967	JX010443
C. queenslandicum	*Carica papaya*	Australia	JX010276	JX009934	JX010414
	Coffea sp.	Fiji	JX010185	JX010036	JX010412
C. salsolae	*Salsola tragus*	Hungary	JX010242	JX009916	JX010403
C. siamense	*Persea americana*	Australia	JX010250	JX009940	JX010387
	Coffea arabica	Thailand	JX010171	JX009924	JX010404
C. theobromicola	*Stylosanthes guianensis*	Australia	JX010291	JX009948	JX010381
	Theobroma cacao	Panama	JX010294	JX010006	JX010447
C. ti	*Cordyline australis*	New Zealand	JX010267	JX009910	JX010441
C. tropicale	*Litchi chinensis*	Japan	JX010275	JX010020	JX010396
	Theobroma cacao	Panama	JX010264	JX010007	JX010407
C. xanthorrhoeae	*Xanthorrhoea preissii*	Australia	JX010261	JX009927	JX010448

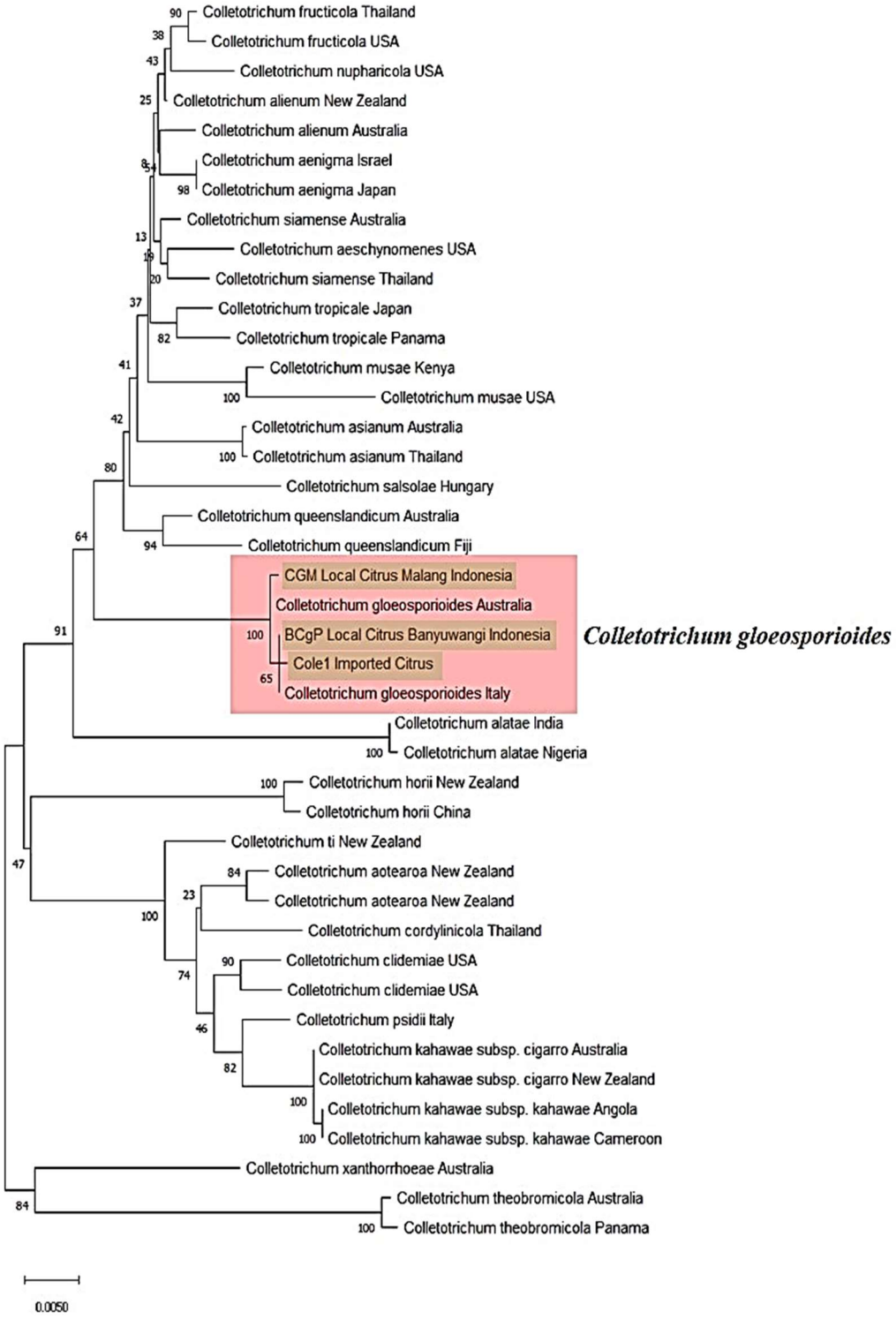

Figure 4. Phylogenetic tree of *C. gloeosporioides* strains using DNA multi locus (ITS, GAPDH, TUB2) from imported and local citrus fruits, and other countries.

Pathogenicity Test

The pathogenicity test showed that *C. gloeosporioides* from imported citrus fruits can cause anthracnose rot disease in imported and local healthy citrus fruits, either through the culture attachment method or the conidial suspension injection method. These results proved that *C. gloeosporioides* carried by imported citrus fruits was a fungal pathogen. *C. gloeosporioides* caused a disease incidence of 100% in imported (Table 7) and local citrus fruits (Table 8). This means that all the tested citrus fruits were rotten. However, the percentage of disease severity showed a different value between the culture attachment method and the conidial suspension injection method. The culture attachment method had a lower disease severity value compared to the conidial suspension injection method. Rot symptoms were seen in 0.25 part of the culture attachment method. Fig. 5 showed that the disease with rot symptoms occurred in almost all citrus fruits by the conidial suspension injection method. *C. gloesporioides* isolated from imported citrus fruits caused high disease severity in both imported and local citrus fruits of 97.78 ± 1.92 and 98.89 ± 1.92, respectively. In addition, *C. gloeosporioides* isolated from local citrus fruits caused a disease severity of 96.67 ± 3.33 (Table 8). Meanwhile, citrus fruits still looked healthy without rotting symptoms at 21 DAI in the control treatment using PDA media attachment without *C. gloeosporioides* and the sterile water injection method.

Table 7. Incidence and severity of anthracnose rot disease in imported citrus fruits

Treatment	Disease incidence (%)	Disease severity (%)
The attachment of blank PDA media without *C. gloeosporioides* (Control)	0 ± 0.00 a	0 ± 0.00 a
The attachment of *C. gloeosporioides* culture isolated from imported citrus fruits	100 ± 0.00 b	38.89 ± 3.85 b
The injection of sterile water without *C. gloeosporioides* (Control)	0 ± 0.00 a	0 ± 0.00 a
The injection of conidial suspension of *C. gloeosporioides* isolated from imported citrus fruits	100 ± 0.00 b	97.78 ± 1.92 c

Values are stated as mean ± SD. Mean values in the same column followed by different letters indicate a significant difference according to Tukey's test at $P < 0.05$.

Table 8. Incidence and severity of anthracnose rot disease in local citrus fruits

Treatment	Disease incidence (%)	Disease severity (%)
The injection of sterile water without *C. gloeosporioides* (Control)	0 ± 0.00 a	0 ± 0.00 a
The injection of conidial suspension of *C. gloeosporioides* isolated from imported citrus fruits	100 ± 0.00 b	98.89 ± 1.92 b
The injection of conidial suspension of *C. gloeosporioides* isolated from local citrus fruits	100 ± 0.00 b	96.67 ± 3.33 b

Values are stated as mean ± SD. Mean values in the same column followed by different letters indicate a significant difference according to Tukey's test at $P < 0.05$.

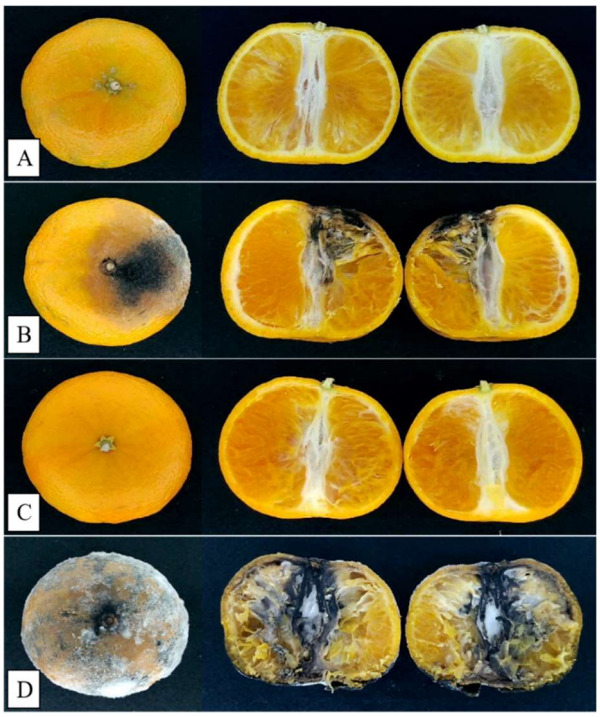

Figure 5. Severity of anthracnose rot disease in imported citrus fruits. A – Healthy citrus fruit in the control treatment of the attachment of blank PDA media without *C. gloeosporioides*; B – Citrus fruit with anthracnose rot disease in the treatment of the attachment of *C. gloeosporioides* culture isolated from imported citrus fruits; C – Healthy citrus fruit in the control treatment of the injection of sterile water without *C. gloeosporioides*; D – Citrus fruit with anthracnose rot disease in the treatment of the injection of conidial suspension of *C. gloeosporioides* isolated from imported citrus fruits.

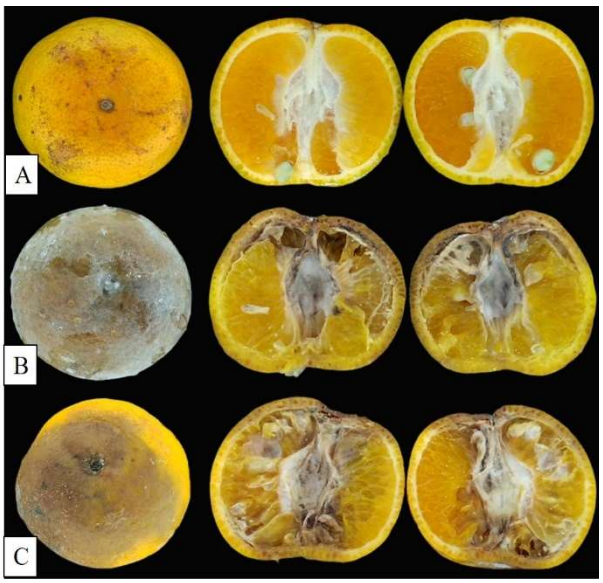

Figure 6. Severity of anthracnose rot disease in local citrus fruits. A – Healthy citrus fruit in the control treatment of the injection of sterile water without *C. gloeosporioides*; B – Citrus fruit with anthracnose rot disease in the treatment of the injection of conidial suspension of *C. gloeosporioides* isolated from imported citrus fruits; C – Citrus fruit with anthracnose rot disease in the treatment of the injection of conidial suspension of *C. gloeosporioides* isolated from local citrus fruits.

Discussion

The ONFIT method was successful in detecting the presence of *C. gloeosporioides* by emerging mycelia on the surface of imported citrus fruits at 4 DAI (Fig. 1). The emergence of mycelia proved that *C. gloeosporioides* was already inside healthy citrus fruit when being imported from the country of origin into Indonesia. According to Michailides & Elmer (2000), *C. gloeosporioides* existed in citrus fruits since the plants

entered the flowering and fruiting phases in the field. The condition of the imported citrus fruits containing *C. gloeosporioides* still looked healthy and fresh without showing symptoms of anthracnose when being arrived at the port of Tanjung Priok, Jakarta. This was due to the status of *C. gloeosporioides* as a latent pathogen with inactive structures or as a non-pathogenic endophyte in citrus fruit tissues (Sinclair, 1991). *C. gloeosporioides* can actively infect when the structure of citrus fruit begins to weaken, either due to senescence or other physical processes (Paramasivan et al., 2009). Faisal et al. (2011) stated that the cause of *C. gloeosporioides* being in a latent position was due to the unripe citrus fruits during harvest and low-temperature storage during the transportation process. Mehrota (2001) explained that unripe fruit is difficult to be infected because the potentially important enzymes possessed by *C. gloeosporioides* are not sufficient to destroy the structural strength of the fruit. Prusky & Lichter (2007) added that unripe fruit cannot provide nutrients that can be directly absorbed by *C. gloeosporioides*. In addition, secondary metabolites in unripe fruit are toxic which can inhibit *C. gloeosporioides* infection.

The ONFIT method worked by destroying the physical and chemical structure of citrus fruits. Agrios (2005) mentions that physical structure is an important factor for plants that can act as a means of host defense against pathogen attack. The plant cell wall is one of the physical defenses. Pathogens generally must first penetrate the plant cell wall to be able to carry out the infection process. The ONFIT method can damage plant cell walls, making it easier for *C. gloeosporioides* to infect and grow on citrus fruits. Ginzberg & Stern (2016) reported the structure of the cell wall of citrus fruits is composed of epidermal and cuticle cells which make the fruit difficult to be infected by pathogens. However, the ONFIT method resulted in the death of epidermal and cuticle cells, making it easy for *C. gloeosporioides* to penetrate. In addition, the ONFIT method is thought to be able to break down the macromolecular structure of complex sugars into a micromolecular structure of simple sugars that can be absorbed directly by *C. gloeosporioides* as a source of nutrition.

Table 2 shows none of the *C gloeosporioides* mycelia growing on 100 citrus fruits in the control treatment. All citrus fruits still looked healthy, fresh, and orange in color (Fig. 1). Health and freshness indicate that citrus fruits are still actively carrying out metabolic processes even though they have been harvested. Brizzolara et al. (2020) explained that citrus fruits still carry out metabolic processes even though they have been separated from the plant. The ONFIT method can destroy fruit cells, so that metabolic processes are no longer produced. Cell death was indicated by a change in colour from orange to dark brown on the surface of the fruit (Fig. 1). Cells that have died are no longer able to carry out metabolism to produce toxic secondary metabolites. Therefore, *C. gloeosporioides* will become active and easily infect. The mycelia of *C. gloeosporioides* became clearly visible at 4 DAI (Fig. 1). However, the mycelial activation of *C. gloeosporioides* in citrus fruits is strongly suspected to have started before the 4th day. The emergence of mycelia also confirms that the ONFIT method only kills fruit tissue but does not kill *C. gloeosporioides* which infects latent in imported citrus fruits.

The technique of dilution and spreading of conidia on PDA media succeeded in obtaining pure cultures that grew from a single conidium. Choi et al. (1999) stated that the use of pure cultures derived from single spore isolation is the basis for the identification process of fungi. In addition, Goh & Hanlin (1997) also stated that fungal

cultures derived from single spores are important in identifying species through a phylogenetic concept approach based on morphological and molecular characters. Characterization of mycelia and conidia identified the species growing on the surface of imported citrus fruits as *C. gloeosporioides*. The characteristics of the conidia were imperfectly cylindrical in shape with two distinct ends. One end of the conidia had a slender or tapered end and the other end was broadly rounded (Fig. 2). Wang et al. (2021) also explained that the conidia of *C. gloeosporioides* were subcylindrical in shape with uneven sizes. The conidia of *C. gloeosporioides* varied in length and width. The conidia of *C. gloeosporioides* isolated from imported citrus fruits in Table 3 had an average length and width of 15.28 ± 2.08 µm × 4.59 ± 0.40 µm almost the same as the conidia of *C. gloeosporioides* from citrus fruits in Florida isolated by Agostini et al. (1992) with a size of 15.5 ± 0.5 µm × 4.5 ± 0.9 µm. In addition, the yellow conidiomata on PDA media were the same as the conidiomata of *C. gloeosporioides* from citrus fruit in Australia isolated by Wang et al. (2021). Several morphological character similarities that were found simultaneously identified that the fungus mycelia isolated from the surface of imported citrus fruits were indeed *C. gloeosporioides*.

DNA concentration, A260/A280 ratio and extraction time were different between both extraction methods (Table 4). Motkova & Vytrasova (2011) explained that the amount of concentration and purity of DNA is the main criterion in evaluating the quality of DNA. The total concentration of DNA produced by the method of Abd-Elsalam et al. (2003) was the highest compared to the method of Ausubel et al. (2003) (Table 3). According to Desloire et al. (2006), the difference in the chemical composition of the extraction buffer from each method was the main cause of the difference of the DNA concentration. In addition, Cermakova et al. (2021) explained that differences in chemical concentrations amount of extraction buffers also produced differences in DNA concentration. The PCR process succeeded in amplifying the target DNA in both extraction methods even though the concentrations were different. Sikdar et al. (2014) explained that the minimum limit for the concentration of fungal DNA used in the PCR process is 5 ng per PCR reaction. Therefore, both DNA extraction methods can be used in PCR reactions because having concentrations > 5 ng. DNA purity was analyzed from the value of the ratio A260/A280 (Motkova & Vytrasova, 2011). Each extraction method produced a different ratio of A260/A280. According to Sambrook et al. (1989), DNA purity was in the range of A260/A280 ratio between 1.8–2. An A260/A280 ratio below 1.8 indicated too much RNA and an A260/A280 ratio above 2 indicated too much protein contamination. Table 4 shows the method of Abd-Elsalam et al. (2003) had an A260/A280 ratio above 2. This means that the method produced quite a lot of protein contaminants. Meanwhile, Ausubel et al. (2003) produced a lot of RNA because it had an A260/A280 ratio below 1.8. Fig. 3 shows that differences in DNA concentrations affect the visualization of DNA bands produced on 1% agarose gel. DNA band in the extraction method Abd-Elsalam et al. (2003) was thicker compared to Ausubel et al. (2003). The intact DNA produced by both methods was of good quality which was characterized by visualization of a single amplicon of target DNA in each well of the agarose gel without any DNA fragments (smearing). Sambrook et al. (1989) explained that DNA bands that were clearly visible as a single band without DNA fragments (smearing) indicated DNA integrity. Lorenz (2012) explained that smears occur due to differences in the size of the DNA in the agarose gel.

BLAST results showed that *C. gloeosporioides* from imported citrus fruits had the highest DNA sequence similarity with *C. gloeosporioides* in all locus. This DNA similarity identified that *C. gloeosporioides* found in imported citrus fruits was *C. gloeosporioides*. Table 4 presents a list of the top five of GenBank isolates that have the highest DNA similarity with *C. gloeosporioides* isolated from imported citrus fruits. Most of the isolates are *C. gloeosporioides*. However, there are several other isolates in ITS locus identified as *C. siamense*, and *C. fructicola*. Referring to the report of Liu et al. (2013) that the other two identities are still in the *C. gloeosporioides* complex group. Thus, the two isolates will definitely have high DNA similarities with *C. gloeosporioides* because they are in the same group as *C. gloeosporioides* species complex.

The phylogenetic tree in Fig. 4 informs that *C. gloeosporioides* from imported citrus fruits places in the same clade with *C. gloeosporioides* from Italy and Banyuwangi, Indonesia. According to Hassan et al. (2002), the position of species within the same clades indicates the same species. *C. gloeosporioides* from imported citrus fruits is different position with *C. gloeosporioides* from Australia and Malang, Indonesia. Hossain et al. (2021) said species of fungi that are in different clades in the phylogenetic tree show genetic variation between fungal species. A genetic relationship that is different clades indicates differences in strains (Das et al., 2020). Thus, *C. gloeosporioides* carried in imported citrus fruits was the same strain with *C. gloeosporioides* from Italy and Banyuwangi, Indonesia. *C. gloeosporioides* carried in imported citrus fruits had an intraspecific variation with *C. gloeosporioides* from Australia and Malang, Indonesia.

C. gloeosporioides carried by imported citrus fruits was a pathogen. Agrios (2005) describes the pathogenic character based on the ability of the fungus to infect and cause the host to become sick. Table 7 informs that *C. gloeosporioides* was able to make 100% of the tested citrus fruits sick. Diseased citrus fruits were characterized by the appearance of black dry rot symptoms on the outside and inside of the citrus fruit (Fig. 5; Fig. 6). Symptoms are expressions of disease caused by the activity of a virulent pathogen (Ciofini et al., 2022). Pathogen virulence can be determined from the severity of the disease (Mojerlou & Safaie, 2012). This study recorded that *C. gloeosporioides* carried by imported citrus fruits was highly virulent on citrus fruits causing disease severity of up to 97.78% in the conidia suspension injection method. Meanwhile, the severity of the disease in the culture attachment method was 38.89%. The severity of the disease was high in the injection method because a large number of conidia were in sterile water in the fruit, so they could spread with the flow of the liquid into the intercellular spaces in the citrus fruit tissue. The movement of water helps in the process of spreading pathogens (Agrios, 2005). Disease severity in the culture attachment method was lower because the pathogen must move without the aid of water movement to a source of nutrition through the activity of mycelia growth and elongation in citrus fruits. *C. gloeosporioides* isolated from imported citrus fruits was also pathogenic to local citrus fruits. As a result, *C. gloeosporioides* could hazard local citrus fruit production (Fig. 6). Disease severity caused by *C. gloeosporioides* isolated from imported citrus fruits was higher than local strain. Therefore, *C. gloeosporioides* from imported citrus fruits was more virulent than the local. The pathogenicity test used a predetermined concentration of 10^5 conidia mL^{-1} to assess the virulence of *C. gloeosporioides* isolated from imported citrus fruits against imported and local citrus fruits. However, the concentration of conidia certainly varies in citrus fruit under in vivo conditions or in storage.

CONCLUSIONS

C. gloeosporioides has been found on imported citrus fruits. *C. gloeosporioides* sensu stricto was successfully detected using the overnight freezing incubation method and identified morphologically and molecularly. *C. gloeosporioides* from imported citrus fruits was the same strain with *C. gloeosporioides* from Banyuwangi, Indonesia, but it has a genetic variation with *C. gloeosporioides* from Malang, Indonesia. *C. gloeosporioides* from imported citrus fruits was pathogenic to imported and local citrus fruits with a disease incidence value of 100%, and a disease severity value of 97.78% and 98.89%, respectively.

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