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IDENTIFICATION AND CROSS INOCULATION OF *Colletotrichum gloeosporioides* ASSOCIATED IN MANDARIN CITRUS

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ABSTRACT

Colletotrichum spp are important post-harvest pathogens that frequently cause anthracnose and significant quality deterioration in citrus. This study were aimed to identify morphological characteristics and genetic confirmation of Colletotrichum isolates associated with mandarin citrus , as well as to evaluate the cross-inoculation potential of these isolates among five post-harvest commodities. Anthracnose-infected citrus fruits were collected from a local market in Central Java, Indonesia. Fungal isolation was conducted using the single-spore technique, and identification was based on colony and conidial characteristics, followed by PCR amplification with the universal ITS1 and ITS4 primers. Cross-inoculation tests were performed on five post-harvest commodities. Morphological and genetic characteristics indicated that the isolates referred to *Colletotrichum gloeosporioides*, the causal agent of anthracnose in mandarin citrus. Colony characters were white-greyish colour with velvety texture, conidiomata, black spots, concentric rings, and fast-growing group. Conidia were cylindrical with rounded ends, measuring 12.02µm in length and 4.23µm in width on average. ITS amplification yielded a ±570 bp fragment that showed 98% similarity to *C. gloeosporioides* isolates from China, indicating a close relationship with the *C. gloeosporioides* species complex. Cross-inoculation tests showed that *C. gloeosporioides* can cause anthracnose symptoms in tomato, cayenne, pepper, citrus, and mango. The finding highlights that *C. gloeosporioides* was detected on mandarin fruits exhibiting anthracnose symptoms, and the potency of the cross-inoculation assay revealed the broad host range of this fungus.

Keyword: *Colletotrichum gloeosporioides,* citrus antrachnose, identification, genetic analysis, cross-inoculation.

INTRODUCTION

Citrus is one of the most important horticultural commodities worldwide, including in Indonesia, where its productivity continued to increase from 2021 to 2023 (BPS, 2024). As a major fruit crop, citrus plays a significant role in supporting agricultural production and economic value. Despite the increasing production, postharvest losses remain a serious challenge. Improper harvesting practices and inadequate postharvest handling can lead to yield losses ranging from 20% to 50%. Postharvest diseases, in particular, can substantially reduce citrus quality and marketability, thereby negatively affecting overall yield and economic returns (Cheng et al., 2020).

Among postharvest pathogens, *Colletotrichum* spp. are well known as the causal agents of anthracnose, a disease of major scientific and economic importance due to its wide host range and destructive nature (Ciofini et al., 2022). Anthracnose is characterized by the development of sunken, dark, and necrotic lesions on infected tissues, often accompanied by acervuli bearing conidia, which facilitate rapid disease dissemination under favorable environmental conditions, particularly high humidity and warm temperatures (Zakaria, 2021). In citrus, anthracnose infections may occur during both preharvest and postharvest stages, with symptoms often remaining latent until fruit ripening or storage, thereby complicating disease detection and management (Aiello et al., 2015).

Colletotrichum gloeosporioides is frequently reported as one of the most prevalent species infecting tropical fruit crops, including citrus (Dowling et al., 2020; Lima et al., 2011; Vu et al., 2023). This pathogen is responsible for various symptoms such as leaf blight, leaf spot, stem-end rot, premature fruit drop, and postharvest anthracnose in citrus (Vu et al., 2023).

Accurate identification of Colletotrichum species remains challenging because many species share similar morphological characteristics that are highly influenced by environmental conditions. Consequently, morphological traits alone are insufficient for species-level identification, making molecular analysis essential (Dowling et al., 2020; Weir et al., 2012). In addition, cross-inoculation among different fruits and vegetables may occur in both field and market environments, increasing the risk of disease transmission. Previous studies have demonstrated that several Colletotrichum species, including *C. siamense*, *C.*

asianum, *C. gloeosporioides*, and *C. sloanei*, are capable of infecting multiple tropical fruit hosts with varying degrees of pathogenicity (Zhafarina et al., 2021; Suparman et al., 2018). However, updated information integrating molecular identification, morphological characteristics, and cross-infection potential of Colletotrichum isolates from mandarin citrus remains limited.

Therefore, this study aimed to characterize the morphological features of Colletotrichum isolates obtained from mandarin citrus, determine their phylogenetic relationships based on nucleotide sequence analysis, and evaluate their cross-inoculation potential on various postharvest horticultural commodities. The findings are expected to provide updated insights into host–pathogen interactions and support the development of more effective disease management strategies.

METHODOLOGY

Study area and Sampling Collection:

The research was conducted at the Laboratory of Plant Protection, Department of Agrotechnology, Faculty of Agriculture, Universitas Pembangunan Nasional "Veteran" Yogyakarta, Special Region of Yogyakarta, Indonesia. The anthracnose symptoms on mandarin citrus cultivar medan berastagi (*C. reticulata*) was obtained from local market in Klaten, Central Java, Indonesia.

Isolation of *Colletotrichum gloeosporioides*.:

The mandarin citrus cultivar medan berastagi (*Citrus reticulata*) that had indications of anthracnose was cleansed by surface-sterilized with 70% alcohol. Tissue sections measuring 1 cm × 1 cm were excised from the boundary between healthy and symptomatic tissues and subsequently cultured on Potato Dextrose Agar (PDA) medium in Petri dishes. For seven days, the culture was maintained at room temperature. The fungal culture was purified using the single-spore isolation method. The PDA culture was cut into 1x1 cm fragments, and 500 µL of sterile water was added to a 1.5 mL tube to create a fungal suspension. Then it was mixed by the vortex. The suspension was streaked onto fresh PDA medium and incubated at room temperature for 12-24h. A sterile needle was used to remove the germinated conidium under a microscope before it was transferred to a fresh PDA medium and kept at room temperature. For further testing, fungal cultures were re-cultured in a different Petri dish with PDA medium.

Macroscopic and microscopic features analysis :

The morphological characters were analyzed by macroscopic and microscopic features. The parameters of macroscopic were following Zhafarina (2021). They were aerial view, reverse view, colony texture, concentric ring, conidiomata, blackspot and growth rate. For the microscopic parameters were conidial shape and size. The conidial size was measured by choosing 50 random conidia after 14 days of incubation.

Molecular identification :

The isolate of fungi was re-cultured in PDA and incubated for a week at room temperature. After that, the DNA of fungal was extracted using Genomic DNA Mini Kit (Plant) Protocol Geneaid. PCR amplification of the ITS region was performed using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). A total of 50 µL was used for the PCR amplifications, which included 19 µL of DDH2O, 25 µL of PCR mix (RedMix), 2 µL of forward primer, 2 µL of reverse primer, and 2 µL of *Colletotrichum* sp. DNA extract. Amplification was performed using a thermal cycler under the following conditions: an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min. The amplification results were electrophoresed on 2% agarose gel such that a total of 3 µL amplified DNA was inserted in the gel. The gel electrophoresed to 100 volts for 30 minutes and the results were observed using UV transilluminator with 1kb DNA ladder (Thermo Fischer Scientific, Maltham, United States). The resulting PCR product was sent to PT. Genetica Science in Banten, Indonesia for DNA sequence analysis.

Phylogenetic analysis :

The ITS sequences of its fungus were aligned using Clustal W and Bioedit to get a consensus sequence. The alignment was checked manually in Bioedit. The phylogenetic tree was constructed using MEGA 12.0 with the Neighbor-Joining method. The sequence of the fungi was compared with the selected NCBI database for approximate identification using the BLAST algorithm. The DNA sequence homology between the fungal sample and the NCBI database was constructed using MEGA 12.

Cross-inoculation assay:

The species of *Colletotrichum gloeosporioides* from mandarin citrus was re-cultured on PDA petridish and then incubated at room temperature for 7 days. The healthy post harvest commodities were prepared which were untreated and un-waxed to do cross-inoculation assay. The surface of the post harvest commodities was cleansed by surface-sterilized with 70% alcohol. Post-harvest commodities that had been cleaned were placed in a tissue paper-lined plastic box and sprayed with sterile water to keep the relative humidity at 95%. The post harvest commodities were inoculated using a wound approach by pin-pricking them with a sterile needle in the middle portion of the post harvest commodities. Then the mycelial disk of *Colletotrichum gloeosporioides* was placed onto the wound. After harvest, the inoculated post-harvest commodities were incubated in a box with a 12-hour light/dark cycle at 30-35°C.

The post-harvest commodities used in cross-inoculation assay were citrus (*Citrus reticula*), mango (*Mangifera indica*), chayotte (*Sechium edule*), tomato (*Solanum lycopersicum*) and cayenne pepper (*Capsicum annuum*) with five replicates per

commodities. The diseases severity was assessed based on lesion development on the symptoms of the commodities. The formula was following Montri et al (2009)

$$\text{Fruit Length Infected (\%)} = \frac{\text{Lesion length}}{\text{Fruit length}} \text{x } 100$$

The data were examined using analysis of variance ($p<0.05$) with Least Significant Difference (LSD) Test for multiple range test using STAR.

RESULTS AND DISCUSSIONS

Symptoms and morphological features of *Colletotrichum gloeosporioides* on mandarin citrus.

The symptoms observed on mandarin citrus were consistent with those previously reported by Ulilalbab et al (2023), characterized by light brown to dark spots enlarging. The infected fruits exhibited sunken lesions and in more advanced stages, cracks developed at the center of larger lesions, exposing the fruit flesh (Fig 1). Tang et al (2023) reported that *C. gloeosporioides* could emerge anthracnose symptoms and asymptoms on citrus fruit. Accordingly, anthracnose caused by *C. gloeosporioides* on citrus fruit exhibits high infection potential, as delayed symptom expression may lead to substantial postharvest losses due to cross-infection and widespread dissemination.

On PDA medium, *C. gloesporioides* produced white to greyish aerial mycelia and a greenish-grey, flat reverse colony. The mycelial texture was velvety with the formation of concentric ring, blackspot and conidiomata. These colony characteristics are consistent with the descriptions reported by Bandgar et al. (2018), that *C. gloeosporioides* isolates predominantly exhibited creamy white to grey colony coloration on the surface, while the reverse side ranged from dark grey to black with slight variations in shade. The observed variation in colony pigmentation supports the known morphological plasticity of *C. gloeosporioides* under different cultural conditions.

The mean colony growth rate was 0.85cm/day which is classified as rapid fungal growth (Fig.1; Table 1). The highest hyphal extension rate was observed on the second day following transfer of the mycelial disk onto fresh PDA medium. By day 10, the fungal mycelium had completely covered the Petri dish with a diameter of 8.5cm (Fig.2). Colletotrichum growth rate is influenced by a number of variables, including temperature, culture media, and the inclusion of additional compounds. (Salotti et al., 2022). Similar with these finding that *C. gloeosporioides* mycelial growth after 7 days of culture over 7.5 cm of petri dish (Syafitri et al., 2023).

B Microscopic features of *C. gloeosporioides* could produce abundant conidia with cylindrical and rounded on both ends (Fig. 1; Table 1). The average size of this fungal was 12.02µm in length and 4.23µm in width. There was no septate found on *C. gloeosporioides's* type, and inline with the Syafitri et al (2023) reported.

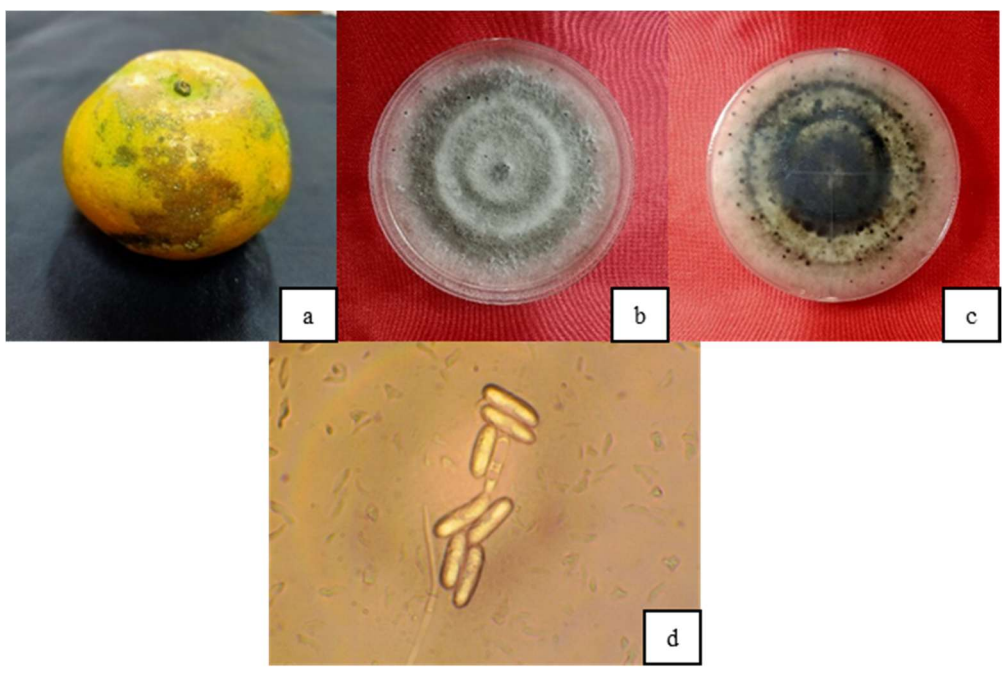

Figure 1. Antrachnose symptoms on mandarin citrus (a); aerial view of *C. gloeosporioides* on PDA(b); reverse view of *C. gloeosporioides* on PDA(c); conidia of *C. gloeosporioides* (Ulilalbab et al., 2023)

Tabel 1. Mycelium and Conidia Features of *Colletotrichum gloesporioides* on Mandarin Citrus

Mycelium and Colony Features	
Aerial view	White greyish
Reverse view	Greenishgrey flat
Colony texture	Velvety
Concentric ring	Present
Conidiomata	Present
Blackspot	Present
Growth rate (cm/day)	0.85
Conidial shape	Cylindrical
Conidial length	12.02µm with range 10.07-14.77µm
Conidal width	4.23µm with range 3.3–5.11 µm

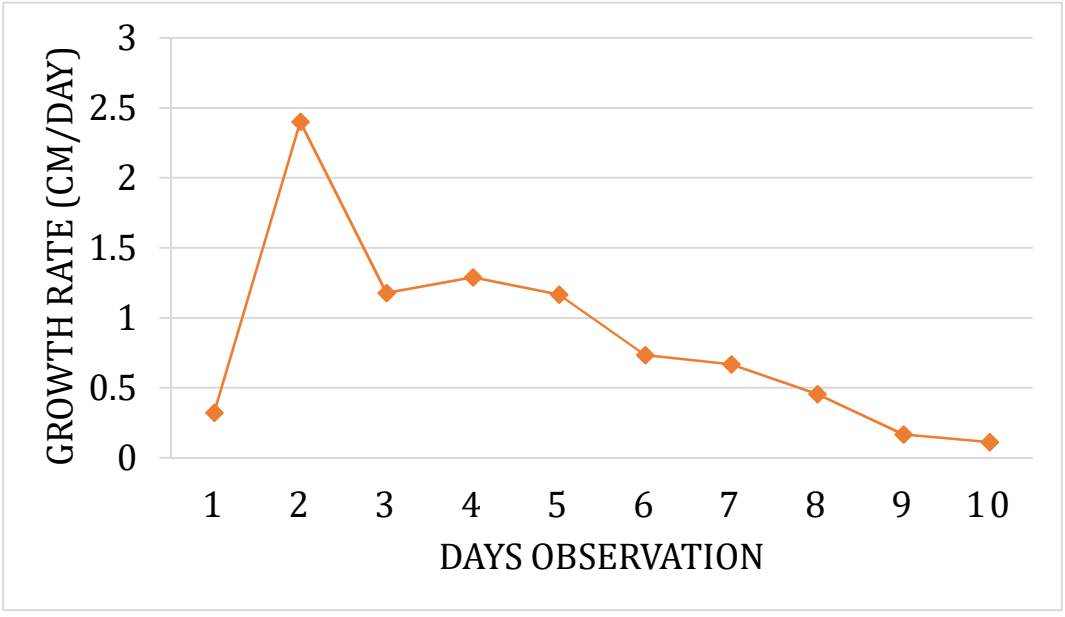

Figure 2. Growth rate of *Coletotrichum gloeosporioides* on PDA medium

Molecular analysis

The primary method of identifying *C. gloeosporioide*s is morphological characteristics, which may result in biased information regarding other Colletotrichum species that may share morphological characteristics. Therefore, molecular identification was needed to evaluate and detect the sample properly. The DNA amplification performed with ITS1/ITS4 with a fragment length ±570 bp as shown in the Fig. 3.

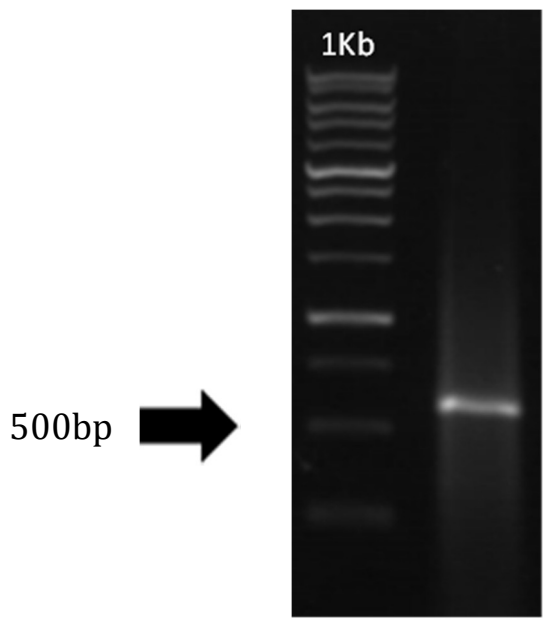

Figure 3. Visual amplification the DNA of *Colletotrichum gloeosporioides* from mandarin citrus with anthracnose symptoms using ITS1/ITS4 with the target ±570bp. (1Kb marker, S : sample)

The results of BLASTn analysis of sequences from the ITS-rDNA region show that isolates have genetic similarity with *C. gloeosporioides* accession code from NCBI MG925324 with a query cover value of 99% and e-value 0.0 respectively. According to Claverie & Notredarme, 2003 A high query value close to 100%; a low E-value close to 0.0 indicate that the query DNA sequence has a high similarity or homology with the DNA sequence in the database.

The isolate's ITS sequence alignment, BLAST alogarithm, and reference species from the NCBI GeneBank database were used to construct the pylogenetic tree. The generally recognized method for identifying *Colletotrichum* species requires phylogenetic analysis and multigene sequencing, with a focus on a polyphasic approach. (Baroncelli et al. 2015; Bragança et al. 2016; Cai et al. 2009; Chechi et al. 2019; Chen et al. 2016; Damm et al. 2012a; Grammen et al. 2019; He et al. 2019; Hyde et al. 2009; Jayawardena et al. 2016a; Liu et al. 2015; Moreira et al. 2019; Park et al. 2018; Wang et al. 2016; Weir et al. 2012; Zhafarina et al., 2021). The phylogenetic tree analysis revealed that the sample was closely related to the China MG925324 *Colletotrichum gloeosporioides*. The sample species belongs to one cluster with JQ366001, JQ366000, KU498257, AJ301908 and AJ301977 as *Colletotrichum gloeosporioides* complex (Nurlaelita et al., 2024).

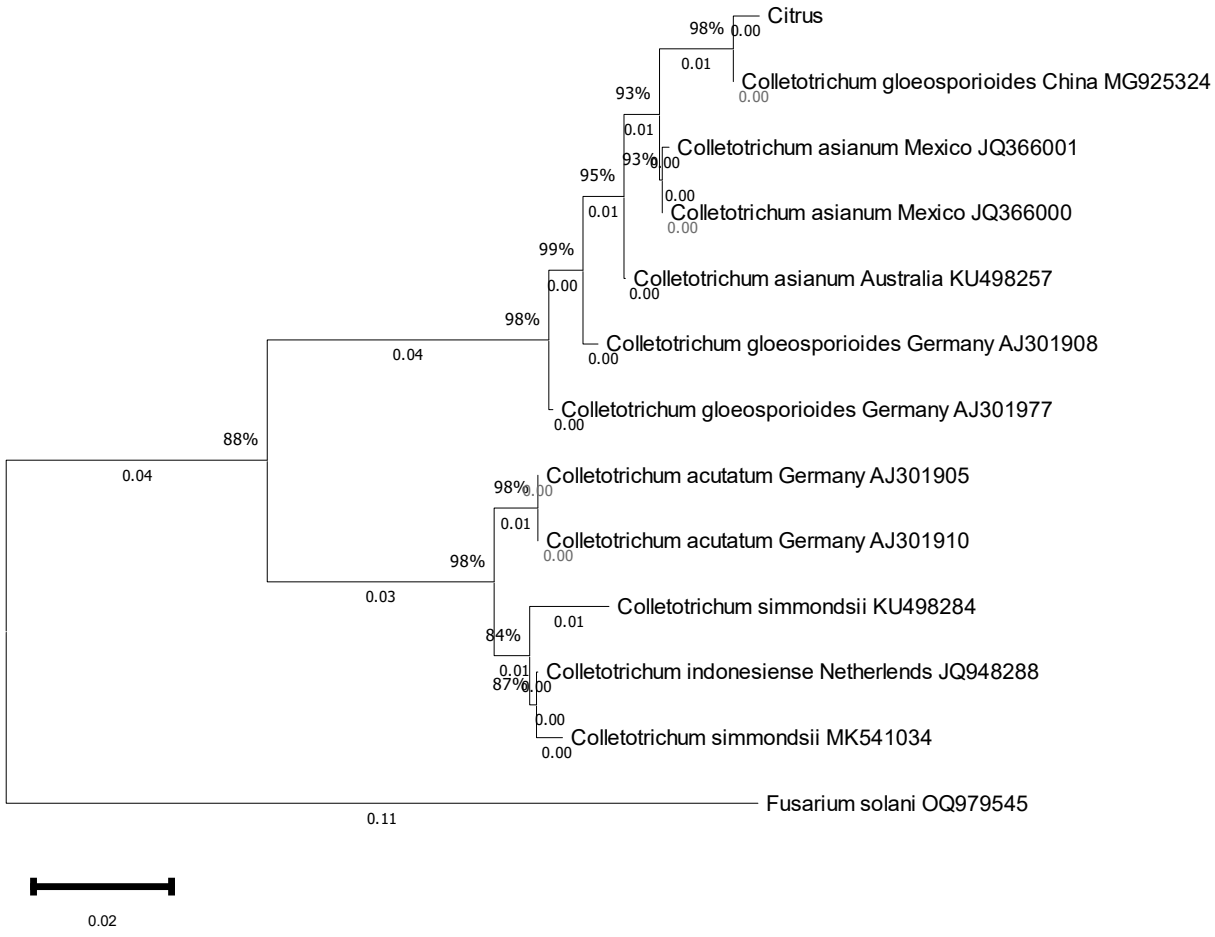

Figure 4. Phylogenetic tree of *Colletotrichum gloeosporioides* from mandarin citrus with other various *Colletotrichum* species complex that had been published by NCBI

Notes : The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

C. gloeosporioides homology analysis showed that the *C. gloeosporioides* nucleotide sequence of mandarin citrus Indonesia had similarities between nucleotide bases of MG925324 *C. gloeosporioides* from China (99.997%), KU498257 *C. asianum* from Australia (99.978%), JQ66001 *C. asianum* from Mexico (99.985%) and AJ301908 *C. gloeosporioides* from Germany (99.972%) (Table 2). This finding indicates a close genetic relationship with *C. gloeosporioides*; however, additional loci would further strengthen species-level resolution.

Table 2. Homology level of *Colletotrichum gloeosporioides* gene nucleotice sequence on mandarin citrus and other database NCBI

Number	Isolates	1	2	3	4	5	6	7	8	9	10	11	12
1	Sample	ID											
2	Colletotrichum_gloeosporioides_China_MG925324	99.997	ID										
3	Colletotrichum_asianum_Australia_KU498257	99.978	99.982	ID									
4	Colletotrichum_asianum_Mexico_JQ366001	99.985	99.987	99.994	ID								
5	Colletotrichum_asianum_Mexico_JQ366000	99.987	99.989	99.996	ID	ID							
6	Colletotrichum_gloeosporioides_Germany_AJ301908	99.972	99.979	99.991	99.985	99.987	ID						
7	Colletotrichum_gloeosporioides_Germany_AJ301977	99.969	99.975	99.987	99.982	99.983	99.993	ID					
8	Colletotrichum_acutatum_Germany_AJ301905	99.891	99.897	99.910	99.902	99.904	99.914	99.920	ID				
9	Colletotrichum_acutatum_Germany_AJ301910	99.891	99.897	99.910	99.902	99.904	99.914	99.920	ID	ID			
10	Colletotrichum_indonesiense_Netherlends_JQ948288	99.907	99.909	99.904	99.904	99.904	99.911	99.915	99.985	99.985	ID		
11	Colletotrichum_simmondsii_MK541034	99.895	99.901	99.901	99.902	99.902	99.910	99.916	99.983	99.983	99.996	ID	
12	Colletotrichum_simmondsii_KU498284	99.885	99.888	99.896	99.884	99.887	99.902	99.907	99.979	99.979	99.988	99.983	ID

Notes : The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). This analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 593 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

Cross-Inoculation Test

The findings of the cross-infection test in this investigation demonstrated the strength of cross-infection between post harvest commodities. The studied isolate could cause symptoms in their native hosts, but not necessarily on additional hosts. The percentage of fruit length infected by *C. gloeosporioides* from mandarin citrus shown the most severe on tomato 72.84% at 8 days after inoculation. Then followed with cayenne pepper 48.22%, mandarin citrus 20.56% and mango 16.67%. The chayote did not show the symptoms of anthracnose (Table 3). Similar findings were reported by earlier research (Lakhsmi et al., 2011; Zhafarina, 2021), however they used different isolates. *C. gloeosporioides* from citrus had the highest percentage of infections on tomato than original host on citrus. According to Zhafarina et al. (2021), the Colletotrichum isolate may be more hostile to other fruits than the original host. Colletotrichum isolates were more aggressive in infecting the host from which they were initially isolated, in contrast to previously documented isolates. A study on cross-infection revealed that different strains of Colletotrichum could infect multiple hosts, and that multiple species of Colletotrichum may infect a single host (Eaton et al., 2021). The ability of the same species to cross-infect different hosts varied, and this should be taken into account while creating new species (Phoulivong et al., 2012).

Incubation period of *C. gloeosporioides* on tomato, cayenne pepper and citrus with wounded inoculation showed the anthracnose symptoms at day 3 after inoculation. Therefore, on mango fruits showed the symptoms at 4 days after inoculation. Observations were made for 8 days to determine the severe symptoms caused by *C. gloeosporioides* (Table 3).

Table 3. Percentage of fruit length infected by *C. gloeosporioides* from mandarin citrus in cross-inoculation test

Potency of *C. gloeosporiodes* inoculation	Fruit Length Infected (%) / day							
	1	2	3	4	5	6	7	8
Citrus	0^{a}	0^{a}	12.53^{b}	15.19^{b}	16.61^{b}	18.20^{b}	20.25^{c}	20.56^{c}
Mango	0^{a}	0^{a}	0.00^{c}	6.00^{bc}	7.07^{bc}	9.61^{bc}	16.27^{c}	16.67^{c}
Chayote	0^{a}	0^{a}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}
Tomato	0^{a}	0^{a}	22.66^{a}	30.09^{a}	38.97^{a}	52.57^{a}	71.83^{a}	72.84^{a}
Cayenne Pepper	0^{a}	0^{a}	20.56^{a}	25.44^{a}	33.37^{a}	36.83^{a}	47.35^{b}	48.22^{b}

*Means with the same letter in each colomn are not significantly different from each other based on LSD

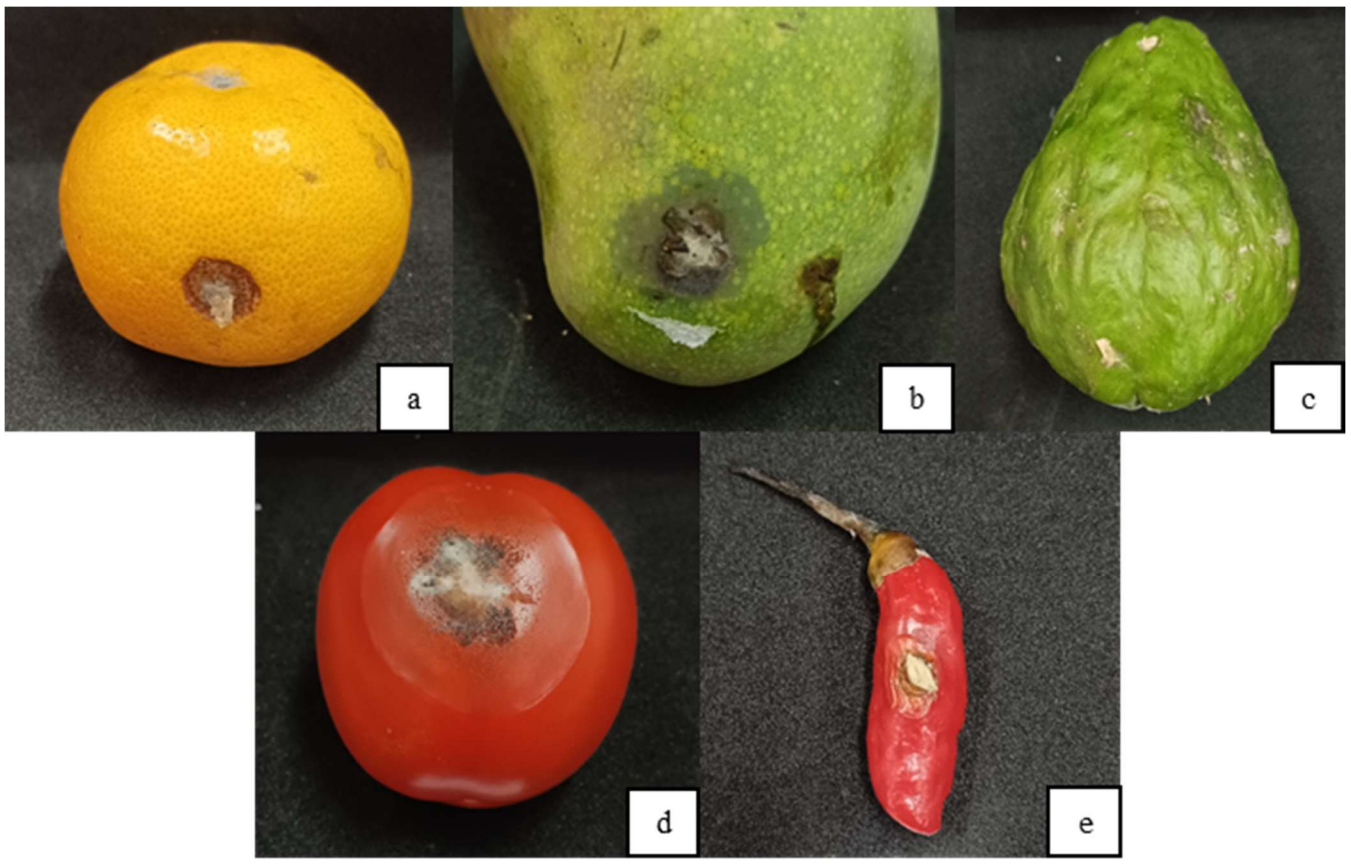

Figure 5. Symptoms of C. *gloeosporioides* on varied hosts/non-host (a) antrachnose symptoms on citrus; (b) mango; (c) chayote, (d) tomato; (e) cayenne pepper.

Cross inoculation assays with *C. gloeosporioides* on detached tomato, cayenne pepper, citrus and mango showed anthracnose symptoms with differences in aggressiveness. Anthracnose symptoms caused by *C. gloeosporioides* varied among host commodities but shared common characteristics. On mandarin citrus (Fig. 5a), symptoms appeared as circular, sunken lesions with brown to dark margins, often expanding and leading to tissue necrosis. Similar sunken necrotic lesions were observed on mango fruit (Fig. 5b), frequently accompanied by tissue collapse and cracking at advanced stages. In contrast, chayote fruit did not exhibit visible anthracnose symptoms during the observation period (Fig. 5c). The absence of external symptoms suggests a possible asymptomatic or latent infection, which has been reported for *C. gloeosporioides* on certain hosts, where the pathogen remains quiescent until favorable conditions trigger disease development (Larralde-Corona et al., 2021). In tomato (Fig. 5d), anthracnose manifested as soft, sunken lesions covered with dark sporulating masses, indicating active conidiation. Meanwhile, chili fruit (Fig. 5e) exhibited elongated, sunken lesions that progressed to tissue shrinkage and rot. Despite host-specific variations, the consistent occurrence of sunken necrotic lesions and sporulation reflects the typical pathogenic behavior of *C. gloeosporioides*, highlighting its broad host range and adaptability across different fruit commodities (Suvama et al., 2015). Overall, our results demonstrated that *C. gloeosporioides* were more aggressive on post-harvest commodities with thin-skinned like tomato and cayenne pepper than citrus and mango (Fig. 5).

CONCLUSION

Genetic and morphological identification in this study refers to *C. gloeosporioides* that caused anthracnose on mandarin citrus in Indonesia. This fungus had the potency of cross-inoculation toward tomato, cayenne pepper and mango although each disease's severity varied in degrees.

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