Identification and Characterization Colletotrichum spp. Causing Mango Dieback in Indonesia

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Dieback disease in mango trees has been observed in Indonesia, particularly in Java Island, with the causal agent remaining unidentified. One of the important pathogens that are responsible for causing mango dieback is Colletotrichum. Field surveys were conducted in various mango cultivating areas in Java Island, Indonesia to assess prevalence of Colletotrichum as dieback disease pathogen. Eleven Colletotrichum isolates were recovered from symptomatic dieback twigs and morphologically characterized. Genetic diversity fingerprint analysis was carried out using rep-PCR. Phylogenetic analysis identified isolates as belonging to Colletotrichum asianum and Colletotrichum cairnsense using partial sequences of four gene regions, including ITS, ACT, GAPDH, and TUB2. Pathogenicity tests on mango seedlings cv. Arumanis showed that all fungal isolates were responsible for causing dieback symptoms. Subsequently, symptomatic tissue was reisolated to fulfill Koch’s Postulate. This study represented new funding for two species of Colletotrichum causing mango dieback in Indonesia.

Keywords: Colletotrichum, dieback, mango

Mango dieback is a significant disease that is affected by several fungal pathogens, including Botryosphaeria dothidea, Diplodia sp., and Ceratocystis sp. (Saeed et al., 2017). According to da Silva et al. (2022), the fungal pathogen that causes mango dieback belongs to the Botryosphaeraceae family and Fusarium genus. Savant and Raut (2000) reported that dieback of mango stone grafts might be attributed to Colletotrichum gloeosporioides Penz. and Botryodiplodia theobromae Pat. The dieback symptoms infected by C. gloeosporioides included the development of dark to brown circular or irregular spots of leaves resulting in elongated black necrotic areas. Also, the development of acervuli was observed on infected shoots or tips of the twig. Additionally, recent discoveries by Hassan et al. (2022) have identified C. gloeosporioides to be fungal pathogen associated with dieback disease in mangoes, as unveiled in a metagenomic nanopore sequencing study. Colletotrichum is widely recognized for inducing mango disease with potential yield losses of up to 100% (Li et al., 2019; Sharma and Shenoy, 2016). In Indonesia, mango dieback cases linked to Colletotrichum infection have been particularly unreported where reviews have predominantly focused on mango fruit anthracnose disease particularly in Java Island, as the mango production center (Benatar et al., 2020). Despite the Colletotrichum affecting various plant parts, significant yield losses still manifest during the fruit-ripening stage when anthracnose symptoms become apparent (Widiastuti et al., 2023). This study aims to identify and characterize Colletotrichum spp. causing mango dieback in Indonesia which needs further concern for biosecurity.

For this study, symptomatic dieback twig samples were purposefully collected from various mango production cen-
The sample had necrosis on twigs, petioles, and leaves, and the necrotic lesion initiated with irregular blackish-brown discoloration, expanding to cause dried areas (Fig. 2A-C).

Infected twig pieces were subjected to disinfection with 70% ethanol for 1 min, followed by 1% sodium hypo-
chlorite (NaOCl) for 1 min. They were then rinsed twice with sterile ddH_2O and dried using sterile filter paper. Two pieces were placed into potato dextrose agar (PDA) medium with 5 μl lactic acid, and the mixture was incubated at 28°C for 3 days. The resulting colonies, representing a single conidium of Colletotrichum, were recultured and incubated under the same conditions (Choi et al., 1999). In this study, 11 Colletotrichum spp. isolates were obtained from different cultivars, consisting of Manalagi, Arumanis, Gadung, Golek, and Gedong Gincu (Table 1).

Genomic DNA was extracted from pure mycelia of Colletotrichum isolates grown in PDA at room temperature 25°C for 7 days using the Taiwan Geneaid Genomic DNA Mini Kit (Plant) (Geneaid Biotech Ltd., New Taipei, Taiwan), following the manufacturer’s protocol. The genetic diversity fingerprints of Colletotrichum spp. were assessed through rep-PCR (repetitive element sequence-based PCR), using two primer sets, including BOX and ERIC. The PCR amplification and visualization method is based on Prahumadipha et al. (2022). PCR mixture consisted of 12.5 μl (dNTPs; MgCl₂; 2× MyTaq HS Red Mix, Bioline, Meridian Bioscience, Cincinnati, OH, USA), 1 μl for each primer (100 μM), 2 μl template DNA isolates, and 8.5 μl ultrapure water, resulting in a total volume of 25 μl. Rep-PCR using BOX (5′-CTACGGCAAGCGACGCTGACG-3′), ERIC1 (5′-ATGTAAGCTCCTGGGATTAC-3′), and

**Table 1. Colletotrichum spp. pure isolates used in this study**

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Host (cultivar of mango)</th>
<th>Province</th>
<th>Subdistrict</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Altitude (m asl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLG-TJB</td>
<td>Manalagi</td>
<td>West Java</td>
<td>Tasikmalaya</td>
<td>–7.2251°S</td>
<td>108.1608°E</td>
<td>347</td>
</tr>
<tr>
<td>MLG-IJB</td>
<td>Manalagi</td>
<td>West Java</td>
<td>Indramayu</td>
<td>–6.3157°S</td>
<td>108.2001°E</td>
<td>11</td>
</tr>
<tr>
<td>GG-IJB</td>
<td>Gedong Gincu</td>
<td>West Java</td>
<td>Indramayu</td>
<td>–6.3085°S</td>
<td>108.1819°E</td>
<td>12</td>
</tr>
<tr>
<td>HM-IJB</td>
<td>Arumanis</td>
<td>West Java</td>
<td>Indramayu</td>
<td>–6.3157°S</td>
<td>108.2002°E</td>
<td>12</td>
</tr>
<tr>
<td>MLG-TJT</td>
<td>Manalagi</td>
<td>Central Java</td>
<td>Temanggung</td>
<td>–7.1638°S</td>
<td>110.0848°E</td>
<td>661</td>
</tr>
<tr>
<td>HM-SDIY</td>
<td>Arumanis</td>
<td>DI. Yogyakarta</td>
<td>Sleman</td>
<td>–7.4607°S</td>
<td>110.2253°E</td>
<td>140</td>
</tr>
<tr>
<td>HM-BDIY</td>
<td>Arumanis</td>
<td>DI. Yogyakarta</td>
<td>Bantul</td>
<td>–7.5015°S</td>
<td>110.2200°E</td>
<td>77</td>
</tr>
<tr>
<td>MLG-PAJT</td>
<td>Manalagi</td>
<td>East Java</td>
<td>Pasuruan</td>
<td>–7.4440°S</td>
<td>113.0134°E</td>
<td>48</td>
</tr>
<tr>
<td>GO-PAJT</td>
<td>Golek</td>
<td>East Java</td>
<td>Pasuruan</td>
<td>–7.4443°S</td>
<td>113.0136°E</td>
<td>49</td>
</tr>
<tr>
<td>GAD-PAJT</td>
<td>Gadung</td>
<td>East Java</td>
<td>Pasuruan</td>
<td>–7.4438°S</td>
<td>113.0134°E</td>
<td>45</td>
</tr>
<tr>
<td>HM-PAJT</td>
<td>Arumanis</td>
<td>East Java</td>
<td>Pasuruan</td>
<td>–7.4439°S</td>
<td>113.0134°E</td>
<td>45</td>
</tr>
</tbody>
</table>

m asl, meter above sea level.

Fig. 3. DNA fingerprints of Colletotrichum spp. causing dieback on mango tree generated with primer sets BOX (A) and ERIC (B) for Colletotrichum spp. Lane 1, MLG-TJB; lane 2, HM-IJB; lane 3, GG-IJB; lane 4, MLG-IJB; lane 5, MLG-TJT; lane 6, HM-BDIY; lane 7, HM-SDIY; lane 8, HM-PAJT; lane 9, GAD-PAJT; lane 10, MLG-PAJT; lane 11, GO-PAJT; left M, 100 bp ladder Geneaid; right M, 1 kb ladder Geneaid.
ERIC2 (5′-AAGTAAGTGACTGGGTGAGCG-3′) was conducted with the annealing temperatures set to 53°C for BOX and 52°C for ERIC. Polymorphic bands, observed between 100 and 3,000 base pairs, were shown in Fig. 3. A dendrogram was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) analysis on rep-PCR based on the presence bands amplified with BOX and ERIC primers in Ntsys 2.2 software. Several isolates represented each banding pattern identical group, and 11 isolates were divided into four major groups based on a similarity coefficient of 70% (Fig. 4). The first group, containing only one fungal, was distinct from others (groups 2, 3, and 4), suggesting that this fungal isolate belonged to different clades of *Colletotrichum* species complex. The representative isolates from each group were chosen for molecular identification. Notably, for the 2nd and 4th groups, multiple isolates were selected to represent the origin of sample accurately. MLG-TJB, HM-IJB, HM-BDIY, MLG-TJT, MLG-PAJT, and HM-PAJT were selected for phylogenetic analysis using partial sequences of four gene regions (internal transcribed spacer [ITS], partial actin [ACT], glyceraldehyde 3-phosphate dehydrogenase [GAPDH], and β-tubulin [TUB2]). Patricia et al. (2021) mentioned that the absence and presence of band information were used to build UPGMA tree to compare the amplification profile of *Colletotrichum* strains. Genetic variability accessed by rep-PCR analysis constructed into a dendrogram phylogenetic also supports the genetic differences among the *Colletotrichum* isolates.

The ITS region, TUB2, ACT, and GAPDH genes, were amplified using set primers of ITS1 (5′-TCCG-TAGGTGACCTGGCG-3′) and ITS4 (5′-TCCTC-CGCTTATGATATGC-3′), BTUB2fd (5′-GTB-CACCTYCARACCGGYCARTG-3′) and BTUB4rd (5′-CCRGAYTGRCRAARACRAAGTTGTC-3′), ACT512F (5′-ATGTGCAAGGCCGGTTTCGC-3′) and ACT783R (5′-TAGAGTCCTTCTGGCCCAT-3′), GDF1 (5′-GGCGTCAACGACCCCTTTCATTGA-3′) and GDR1 (5′-GGGTGAGTGTACTTGAGCATGT-3′). PCR amplification and visualization adjustment were performed based on Wang et al. (2021), with annealing temperatures were set to 52°C, 58°C, 60°C, and 55°C for ITS, ACT, GAPDH, and TUB2, respectively. The results of PCR amplification on six isolates showed that all isolates amplified in the range of 600 bp for the ITS and 500 bp for the target β-tubulin region. Meanwhile, the target areas of the Actin and GAPDH were amplified in the range of 300 bp. The phylogenetic tree was constructed from concatenated ITS, ACT, GAPDH, and TUB2 sequences of *Colletotrichum* spp. samples and reference *Colletotrichum* isolates from GenBank (Table 2) using the maximum likelihood method with the Kimura 2-parameter model, and it was tested by 1,000 bootstrap replications.

The phylogenetic tree (Fig. 5) showed that six representative isolates formed two main groups with a high bootstrap value exceeding 50%. HM-BDIY, MLG-PAJT, and HM-PAJT had close relations to *Colletotrichum asianum* CMM4067, while HM-IJB and MLG-TJT clustered with

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**Fig. 4.** Dendrogram resulting from Unweighted Pair Group Method with Arithmetic Mean cluster analysis of DNA banding profiles generated with primer sets combined of BOX and ERIC for *Colletotrichum* spp. causing dieback on mango. The isolates in the red sign were selected for phylogenetic analysis.
C. asiaticum CMM4056. On the other hand, MLG-TJB was separated and clustered with *Colletotrichum cairnsense* AC11. It was observed that *C. asiaticum* belonged to *Colletotrichum gloeosporioides* species complex, whereas *C. cairnsense* belonged to *Colletotrichum acutatum* species complex (de Silva et al., 2017). The identification was in line with the genetic fingerprint results obtained from rep-PCR. The sequences of the six isolates (24 sequences in total) have been deposited in GenBank with the following accession numbers, ITS: OR945809-OR945814, actin: PP091732 to PP091736 and PP091747, GAPDH: PP091737 to PP091741 and PP091748, TUB2: PP091742 to PP091746 and PP091749.

Macroscopic and microscopic morphological observations were conducted on 10-day-old colonies. In general, *Colletotrichum* isolates produced light to dark olive-grey mycelium, with the center of colonies covered by orange conidia masses (Uysal et al., 2022). This study recorded a mycelial growth rate of 7.24 mm/d to 7.89 mm/d, as shown in Table 3. *C. asiaticum* had cottony mycelium, predominantly white, occasionally white grayish with sparse aerial hyphae (Fig. 6A). In this study, isolates identified as *C. asiaticum* exhibited morphological characteristics that closely matched those described by Benatar et al. (2020). The authors used the morphology of the MLG-TJT to represent *C. asiaticum* isolates in this figure, as they were the closest to the reference Meanwhile, *C. cairnsense* had white-greenish cottony mycelium (Fig. 6F). De Silva et al. (2017) also mentioned that *C. cairnsense* had a pale white-grey to olivaceous grey mycelium with a whitish margin. The reverse side of both species was white, typically forming concentric rings and dark spots as mature conidiomata developed (Fig. 6B and G). Additionally, both species produced masses of conidia (conidiomata) as presented in Fig. 6C and H.

Microscopic characterization was observed using an Olympus CX31 microscope (Tokyo, Japan) and Micons Optilab (PT Miconos, Yogyakarta, Indonesia) and it
showed that *C. asianum* and *C. cairnsense* produced hyaline, smooth-walled cylindrical conidia with distinguishing features. For instance, *C. asianum* had obtuse ends, and the conidia of *C. cairnsense* had acutely rounded ends (Fig. 6D and I). The length and width of the conidia were measured by randomly selecting 50 conidia using a calibrated ImageRaster software (Ramos et al., 2016). Conidia measured 7-18.6 μm in length, 2.3-6.9 μm in width, and had a length/width ratio of 2.56-3.23 (Table 3). The formation of appressoria was observed using slide culture technique by growing the isolates on PDA (Siddiquee, 2017). Both *C. asianum* and *C. cairnsense* formed appressoria originating from mycelia, characterized by dark brown, ovoid conidia with irregular shapes and often became complex with age. In contrast, de Silva et al. (2017) reported that *C. cairnsense* formed appressoria single that was medium brown, smooth-walled, subglobose, ovoid to ellipsoidal. In this study, only *C. asianum* produced setae that were perpendicular in shape and dark brown (Fig. 6E and J). *C. asianum* was initially reported in Indonesia by Benahtar et al. (2020) in connection with mango anthracnose in Indramayu. On the other hand, *C. cairnsense* has not been previously recorded in the country but was first identified in Australia. *C. cairnsense* sp. nov. was recognized as the causative agent of chili anthracnose by de Silva et al. (2017). According to the morphology observed in this study. The conidia of *Gloeosporioides* species complex were cylindrical with obtuse ends, while *C. acutatum* species complex had cylindrical conidia with two acute ends or one end slightly obtuse.

Pathogenicity test was conducted on 8-month-old mango seedlings, specifically cv. Arumanis, with an average height of approximately 50 cm per seedling. cv. Arumanis were selected for this pathogenicity test because these cultivars are widely cultivated in Indonesia and frequently show
dieback symptoms due to *Colletotrichum* infection, despite the lack of official reports documenting these cases. The test was performed exclusively on representative isolates identified through phylogenetic analysis, and all isolates of both *C. asianum* and *C. cairnsense* showed pathogenicity (Fig. 7B and D). Moreover, inoculation followed the method outlined by Mayorquin et al. (2019) with modifications. An active hyphal plug (approximately 5 mm in diameter) from a 14-day PDA culture of *Colletotrichum* spp. was placed on the twig tip, creating wounds up to 3 mm in diameter and 2 mm in depth, and covered with a moist sterile tissue then sealed. Control treatments were carried out by placing a sterile plug on the seedling with identical wounds. This process was repeated four times and incubated at greenhouse temperatures (approximately 28°C). A necrotic lesion was observed behind dieback lesion (Fig. 7C and E), with an orange-to-black conidial mass visible on the necrotic area (Fig. 7D). The control twig showed no symptoms (Fig. 7A), and the virulence assessment measured the lesion area on the 20th day after inoculation. All six representative isolates had virulence, with TJT-MLG (188 mm²) and PAJT-HM (119 mm²) identified as the most aggressive isolates (Fig. 7G).

This study found that *C. asianum* was in line with several observations, indicating the isolates were considered the most significant fungal associated with mango diseases. According to Benatar et al. (2020), *C. asianum* was a virulent pathogen causing mango anthracnose in Indramayu, Indonesia. Vitale et al. (2020) also mentioned that *C. asianum* was a common species responsible for mango anthracnose, reported in various countries such as Australia, Brazil, China, Ghana, Japan, Malaysia, Mexico, Panama, Philippines, South Africa, Sri Lanka, Thailand, and Florida. Other species in *C. acutatum* species complex, particularly *C. cairnsense*, were newly recognized causes of mango dis-

Table 3. Characteristics of conidia and mycelial growth

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Conidia length × width (μm)</th>
<th>L/W ratio</th>
<th>Mycelial growth (mm/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLG-TJB</td>
<td>(7-16.1) ± 1.94 × (2.8-5.1) ± 0.54</td>
<td>2.79</td>
<td>7.24 ± 0.09</td>
</tr>
<tr>
<td>HM-IJB</td>
<td>(7.8-13.3) ± 1.31 × (2.9-5.2) ± 0.51</td>
<td>2.79</td>
<td>7.60 ± 0.11</td>
</tr>
<tr>
<td>MLG-TJT</td>
<td>(10.9-16.4) ± 1.11 × (3.8-6.9) ± 0.58</td>
<td>2.74</td>
<td>7.30 ± 0.13</td>
</tr>
<tr>
<td>HM-BDIY</td>
<td>(10.8-14.6) ± 0.97 × (2.9-5.5) ± 0.58</td>
<td>2.93</td>
<td>7.89 ± 0.01</td>
</tr>
<tr>
<td>MLG-PAJT</td>
<td>(9.2-18.6) ± 1.79 × (3.8-6.6) ± 0.74</td>
<td>2.56</td>
<td>7.78 ± 0.02</td>
</tr>
<tr>
<td>HM-PAJT</td>
<td>(8.6-15.3) ± 1.48 × (2.3-5.6) ± 0.64</td>
<td>3.23</td>
<td>7.69 ± 0.02</td>
</tr>
</tbody>
</table>

*Conidia length and width were measured on 50 conidia for each isolate. The data are mean ± standard deviation.*

*Length per width.*

*Mycelial growth is measured in four perpendicular ways.*
eases, despite previously being identified to be pathogens on Chili in Australia.

In conclusion, dieback studies associated with *Colletotrichum* infection were significantly scarce, but this study provided a new examination of mango dieback in Indonesia. It represented the new documentation of *C. asianum* and *C. cairnsense* as the causative pathogens. Further investigations were required to explore and quantify the occurrence and intensity of mango dieback caused by *Colletotrichum* in Indonesia. Accuracy in diagnosing causal pathogens was crucial for the efficacy of disease management.

**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

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Colletotrichum of Mango Dieback