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Original Article

Characterization and pathological diversity of *Colletotrichum* species associated with anthracnose disease on mango in Peninsular Malaysia

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Abstract

Colletotrichum is one of the important postharvest pathogens to cause anthracnose, which is a threatening disease for mango in Malaysia. The information regarding pre-harvest anthracnose disease on mango in Malaysia is still inadequate, therefore encouraging the commencement of this study. The objectives of this study are to identify fungi species from mango anthracnose disease, and to determine the pathogenicity of *Colletotrichum* isolates obtained from the infected mango. During a series of sampling in July 2014 to May 2015 throughout Peninsular Malaysia, the symptom of anthracnose disease was observed in the Malaysian mango plantation. There were 33 isolates of *Colletotrichum* species were purified and successfully identified as *Colletotrichum gloeosporioides* species complex. The identity of the isolates was confirmed and classified into *C. gloeosporioides* (15 isolates) and *C. asianum* (18 isolates). For pathogenicity test using a non-wounded method, the mango was inoculated with a young mycelial disk. Disease symptoms were observed as a brown to black circular or irregular shape of the lesion with the sunken effect on the infected fruits. *Colletotrichum asianum* R2262 appeared as the most pathogenic isolate with DSI of 50% on day 8 after inoculation. The pathogens identified in this study were successfully re-isolated from all the symptomatic mango tissues that resulted in fulfilling the Koch's postulates. Meanwhile, control mango inoculated with non-colonized PDA plugs remained symptomless until the end of the test. The data obtained from this study is crucial to design an effective strategy to control anthracnose disease of mango.

Keywords: *Colletotrichum*, Mango, Internal transcribed spacer (ITS), Malaysia, Anthracnose

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Introduction

Anthracnose disease caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. (Fitzel and Peak, 1984) and *Colletotrichum acutatum* J.H. Simmonds (Freeman et al., 1998) able to attack in pre- and post-harvest stages. Anthracnose disease has reportedly infected several other crops such as banana, avocado, papaya, coffee, passion fruit, guava, dragon fruit and chilli (Anuar et al., 2013; Masyahit et al.,

2009; Than et al., 2008). The capability of *Colletotrichum* to infect during pre- and post-harvest phases can reduce mango production and create terrific loss in the economics of a country. Previous studies have well documented them as severe post-harvest diseases (Jianyou et al., 2018; Kamle and Kumar, 2016; Giblin et al., 2018). Nevertheless, this current study had improved the information regarding the pre-harvest disease of mangoes associated with anthracnose and fruit rot diseases in Malaysia.

According to Awa et al. (2012), the disease of anthracnose has been commonly found associated with mango fruits produced in the humid forest region with the disease incidence that can achieve 100% (Arauz, 2000). Although mango's flowering season occurring in constantly dry weather conditions is able to stimulate huge amount of mango blossom development, the interfering of rain provides a conducive environment for *Colletotrichum* species infection.

In this study, internal transcribed spacer (ITS) region was used for fungal species confirmation because it has been regarded as a universal and barcode region for fungi and found reliable in all eukaryotic organisms. In addition, uses of multiple gene sequence may resolve the taxonomic confusion among fungal that contained species complex. In order to verify the *Colletotrichum* species complex as a causative agent, pathogenicity tests were done repeatedly to ensure similar result obtained.

Information on specific plant diseases plays a vital role in developing plant disease control. Plant disease management or previously known as plant disease control is constructed with the goal to reduce the economic and yield loss besides aesthetic damage caused by plant disease. One of the disease management approaches is the use endophytic bacteria to promote growth and yield of plant (Gholami et al., 2013). Therefore, in order to achieve the target, accurate diagnosis of the disease is essential to identify the pathogen, which is the real target of any disease management program. Moreover, a comprehensive understanding about disease cycle, climatic and environmental factor should also be considered to produce effective plant disease management (Maloy, 2005; Prasetia et al., 2018).

This study will provide the additional knowledge regarding pre-harvest disease of mango associated with anthracnose and fruit rot in Malaysia. Gathering appropriate information on the morphological and molecular identity and pathogenicity test of *Colletotrichum* species will contribute a better-integrated disease management and improve mango production in country. The objectives of this study were to isolate and identify fungal cultures isolated from pre-harvest anthracnose disease on mangoes as well as determining whether or not the isolated *Colletotrichum* species are pathogenic.

Material and Methods

Sampling, isolation and purification of fungal isolates

Sampling locations of anthracnose infected samples were located in several orchard locations throughout Peninsular Malaysia. The locations covered in this study were Perlis, Penang, Perak, Selangor, Pahang and Melaka states. The sampling series was conducted from July 2014 to May 2015. For each sampling site, five samples of fruits and leaves showing the symptom of anthracnose disease and fruit rot were collected.

Potato dextrose agar (PDA) was used in fungal isolation. Samples with anthracnose symptoms were washed with running tap water. Fungal isolation from fruits and leaves of infected samples were performed using method described by Photita et al. (2005) in the absence of visible sporulation. Three of 5 x 5 mm^2 pieces from the margins of infected tissues were taken and surfaced sterilized in 0.5% sodium hypochlorite solution by dipping for 3-5 min and rinsed three times with sterilized distilled water. The tissues were placed on the surface of PDA plate after blot dried with sterile filter paper. The plates were incubated at room temperature (27±1°C) and observed periodically.

The fungal mycelia developed from infected tissues were scraped and transferred onto 4% water agar (WA) using streaking technique to achieve single-spore colonies (Nor Azizah et al., 2015). The plates were incubated at 27±1°C for 24 hours. To obtain a pure culture, the growing single spore or hyphae tip was cut and transferred to PDA using sterilized needle and incubated at 27±1°C for 5 days.

Morphological characterization

All purified isolates were directly observed on colony features, pigmentation and micro-morphological characteristics of the isolates after seven days of incubation. PDA was used to observe and record the macro-morphological characteristics of cultures such as colony morphology, pigmentation and growth rate. For micro-morphological characteristics, WA was used to prepare slide culture and to initiate new

colonies from hyphal tip isolation. The micro-morphological identification was performed by culturing the pure isolates into WA with modification of de Oliveira Costa et al. (2010).

Internal transcribed spacer (ITS) sequence analysis

All pure isolates were cultured on PDA and incubated for 7 days at 27±1°C with 12 h photoperiod. Genomic DNA of the isolates was extracted using UltraClean ® Microbial DNA isolation kit (MO BIO, Carlsbad, CA, USA) following the instruction manual. Polymerase chain reaction (PCR) amplification of ITS region was carried out using the primers of ITS1 (5'-TCCGTA GGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR mixtures for the reactions were performed using GoTaq® Flexi DNA Polymerase (Promega, USA). Amplification of ITS region was referred to White et al. (1990) with slight modification for *Colletotrichum* isolates. Amplifications were performed using Biometra (T Professional) and to verify the absence of any non-specific reaction and contaminants, one control reaction with no DNA was replaced with distilled water.

The amplicons of ITS were observed between 500-600 bp. The amplicons were separated by electrophoresis using 1.5% agarose gels in 1.0X Tris Borate-acid EDTA (TBE) buffer amended with FloroSafe DNA stain according to manufacturer's instructions (1st BASE, Asia). The gel was viewed and analyzed using Syngene software by a gel documentation system under UV light visualization (Syngene, Germany).

PCR products were purified using the Gel Purification Kit according to Qiagen's instruction. The purified ITS products were sequenced in both directions using an Applied Biosystems 3730xl DNA Analyzer at MyTACG Bioscience Company, Malaysia. The ITS sequences obtained were aligned using Molecular Evolutionary Genetics Analysis (MEGA 6.0) (Tamura et al., 2013). The aligned sequences were undergone nucleotide analysis using Basic Local Alignment Search Tool (BLASTn) to find the similarity and compared with the established sequences in GenBank database (https://blast.ncbi.nlm.nih.gov).

Pathogenicity test

Matured and healthy mango fruits (cv Chokanan, MA224) that were uniform in size and age harvested from an orchard in Melaka were used for the pathogenicity test. They were washed with running tap water and surface disinfected with 0.5% sodium hypochlorite (NaOCl) for 5 min, and then rinsed twice with sterile distilled water (Than et al., 2008). After air dried, the fruits were placed in surface sterilized plastic containers (30 x 20 x10 cm) and prepared for inoculation.

Each fruit was inoculated using non-wounded method by placing it directly to 5 mm diameter of mycelium plug on the mango surface (Kouame et al., 2010; Marques et al., 2013). All the inoculated mangoes were incubated in covered containers at same condition, 27 ± 1°C in the dark. All treatments and control were repeated twice and four mangoes were used for each fungal isolate. After eight days of inoculation, the fungal colonies from lesion were re-subcultured onto PDA. The isolated fungal were tentatively re-identified. Those isolated fungal were compared with the original isolates to fulfill the Koch's postulates. Similar cultures obtained with inoculums were confirmed their pathogenicity. Pathogenicity of the isolates was evaluated based on a disease scale from 0 to 4 as described by Amadi et al. (2009) with a modification for mango.

To compare the variation of the disease severity index (DSI) distribution among isolates, the data were analyzed using the Friedman Test of the non-parametric test of the SPSS program at $p<0.05$. Meanwhile, the differences in lesion length caused by each species were determined by two-way ANOVA and means were compared with LSD test at 5% significance level.

Results and Discussion

Identification of fungi species isolated from mango anthracnose disease

The symptom of anthracnose disease was observed in the Malaysian mango plantations. The infected mangoes showed a dark brown to black circular or irregular form of the disease lesion with the sunken effect were detected on the local commercial varieties such as Chokanan (MA 224), Epel (MA 194), Harum Manis (MA 128), Lemak Manis, Melaka Delight and Telur. A total of 33 isolates of *Colletotrichum* species were successfully isolated and were purified using single-spored isolation technique. Based on morphological characteristics, all the isolates identified as *C. gloeosporioides* species complex.

On PDA, *Colletotrichum gloeosporioides* species complex have white to a grey color on the colony with

some isolates have a presence of grey or brown zonation. Based on further micro-morphological characteristics, 33 isolates in this study were divided into 2 different groups assigned as morphotype 1 and morphotype 2 comprising 15 and 18 isolates, respectively (Table 1).

The characteristics used for morphotype classification were focused on conidial features such as the size, apex and base shape of the conidia, the production of setae and colony pigmentation of isolates. Generally, conidia of *C. gloeosporioides* species is described as one-celled, hyaline, aseptate, straight and cylindrical in shape.

The colony features of morphotype 1 isolates produced abundant white to pale grey of fluffy mycelia with presence of zonation in greyish colour (Figure 1A, C). The pigmentation of these isolates shown occurrence of black spots scattering the plate and the colour were depending on the colony features, as greyish the colony observed, the darker in cream to brown the pigmentation produced in PDA (Figure 1B, D). The presence of setae with the length up to 66 µm (Figure 1E, F) and black irregular appressoria (Figure 1G) were important characteristics in *Colletotrichum gloeosporioides* species identification. Morphotype 1 isolates have obviously conidia with the rounded shape at both apex and base with the length size range from 12.5 to 15 µm (Figure 1H).

Table-1: Morphological characteristics of morphotype 1 and 2 of *Colletotrichum gloeosporioides* species complex associated with mango in Peninsular Malaysia

Group	Isolates	Morphological characteristics								
		Macro-morphology			Micro-morphology					
					Conidia			Appressoria		Seta
		Colony features	Pigmentation	Growth rates (mm/day)	Length	Width	Shape	Shape	Colour	
Morphotype 1 (*C. gloeosporioides*, 15 isolates)	B1477, B1505, B1508, B1519, B1524, B1555, C1550, C1551, C1553, C1864, M1567, M1570, M1692, R2258, R2275	White to pale grey, fluffy colony	Pale orange with grey zonated and black spots	5.33	12.5-15µm	3.75-7.5µm	Cylindrical, rounded at both ends (apex and base)	Irregularly lobes	Black	Present
Morphotype 2 *(C. asianum*, 18 isolates)	B1558, M1678, R1733, R1766, R1808, R2254, R2255, R2257, R2262, R2263, R2265, R2266, R2267, R2271, R2272, R2276, R2278, R2279	White to brownish grey, thin mycelium	Pale orange with black zonated	2.83	20-25µm	2.5-3.75µm	Cylindrical, round apex and pointed base	Absent	Absent	Absent

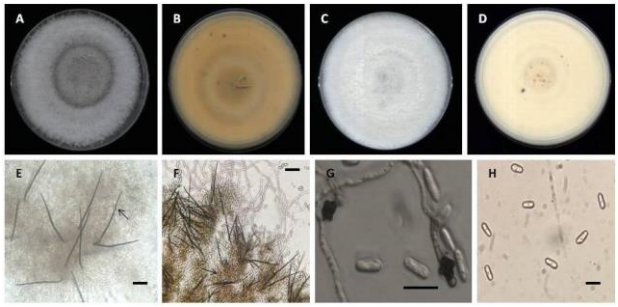

Figure-1: Morphological characteristics of *Colletotrichum gloeosporioides* morphotype 1.
(A)(C) white to pale grey of cottony mycelium with greyish zonation on PDA, (B)(D) zonated cream to brown pigmentation scattered with black spots (E) setae, (F) acervuli, (G) black appressoria, (H) cylindrical conidia with rounded apex and base. Bars =25 µm

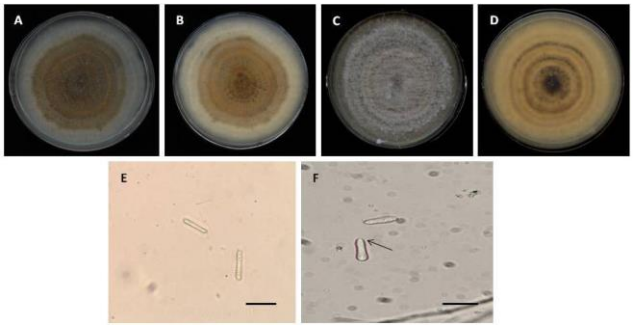

Figure-2: Morphological characteristics of *Colletotrichum gloeosporioides* morphotype 2.
(A)(C) white to brownish grey of thin mycelium with visible orange mass of conidia at centre of PDA plate, (B)(D) pale orange to creamy colour of pigmentation bordered by brown zonation at centre , (E) conidia, (F) conidia with pointed end. Bars = 25µm.

Morphotype 2 isolates showing the colony morphology distinct from morphotype 1 in which the mycelia produced were thin in layer and consist of noticeable orange mass of conidia towards the centre of PDA (Figure 2A-D). The isolates also formed dark zonations that becoming darker at the centre of plate. The difference size of conidia produced in this group was ranged between 7.5 to 10 µm longer in length and 1.25 to 3.75 µm thinner in width (Figure 2E-F). Besides, conidial isolates in this morphotype 2 formed different types of ends shape which are round and pointed at the apex and base of conidia, respectively. The formation of setae, however, was absence in this morphotype.

Conidia of *C. gloeosporioides* species was described as a single-celled, hyaline, aseptate, straight and cylindrical in shape. The morphological characteristics of both morphotypes 1 and 2 fall within the description of *C. gloeosporioides* species complex by Ashraful et al. (2017). The variation between the characteristics in *C. gloeosporioides* species indicated the complexity present known as *C. gloeosporioides* species complex. *Colletotrichum gloeosporioides* species complex or *C. gloeosporioides* sensu lato is a group of *Colletotrichum* species with wider genetic and biological characteristics and share the similarity in conidia features (Weir et al., 2012, Latiffah et al., 2015).

The identity of the isolates was double-confirmed based on molecular characterization. The nucleotide sequences of all isolates amplified by ITS region were aligned and edited using MEGA 6.0 and revealed to have nucleotide length ranged 455 to 626 bp. The sequence of ITS region has finally confirmed with supported of morphological characteristics. The ITS region is the most frequently chosen genetic marker for the molecular identification of fungi in environmental sequencing and molecular ecology studies (Nilsson et al., 2015; Saliha et al., 2018).

Table-2: BLASTn analysis of all 33 isolates associated with anthracnose disease in mango

Isolates	Location (State, City)	Mango variety	Species identification based on ITS		
			Species	Sequence length (bp)	Accession number
B1558	Tanjung Karang, Selangor	Telur	*C. asianum*	556	KT968440
M1678	Telok Mas, Melaka	Melaka Delight	*C. asianum*	554	KT968444
R1733	Arau, Perlis	Chokanan(MA 224)	*C. asianum*	553	KT968443
R1766	Arau, Perlis	Chokanan (MA 224)	*C. asianum*	555	KT968442
R1808	Meru, Selangor	Chokanan (MA 224)	*C. asianum*	521	KT968441
R2254	Kangar, Perlis	Harum manis (MA 128)	*C. asianum*	626	KT968439
R2255	Kangar, Perlis	Harum manis (MA 128)	*C. asianum*	555	KT968438
R2257	Kangar, Perlis	Harum manis (MA 128)	*C. asianum*	553	KT968437
R2262	Beseri, Perlis	Lemak manis	*C. asianum*	553	KT968436
R2263	Kangar, Perlis	Harum manis (MA 128)	*C. asianum*	552	KT968435
R2265	Chuping, Perlis	Harum manis (MA 128)	*C. asianum*	553	KT968434
R2266	Chuping, Perlis	Harum manis (MA 128)	*C. asianum*	552	KT968433
R2267	Chuping, Perlis	Harum manis (MA 128)	*C. asianum*	554	KT968432
R2271	Chuping, Perlis	Harum manis (MA 128)	*C. asianum*	555	KT968431
R2272	Kangar, Perlis	Harum manis (MA 128)	*C. asianum*	553	KT968430
R2276	Chuping, Perlis	Harum manis (MA 128)	*C. asianum*	554	KT968428
R2278	Beseri, Perlis	Harum manis (MA 128)	*C. asianum*	553	KT968429
R2279	Beseri, Perlis	Harum manis (MA 128)	*C. asianum*	554	KT968427
B1477	Meru, Selangor	Epel	*C. gloeosporioides*	555	KT968450
B1505	Meru, Selangor	Epel	*C. gloeosporioides*	558	KT968448
B1508	Meru, Selangor	Epel	*C. gloeosporioides*	553	KT968452
B1519	Meru, Selangor	Telur	*C. gloeosporioides*	552	KT968455
B1524	Meru, Selangor	Telur	*C. gloeosporioides*	552	KT968454
B1555	Meru, Selangor	Epel	*C. gloeosporioides*	552	KT968453
C1550	Maran, Pahang	Telur	*C. gloeosporioides*	555	KT968445
C1551	Maran, Pahang	Telur	*C. gloeosporioides*	547	KT968446
C1553	Maran, Pahang	Telur	*C. gloeosporioides*	548	KT968447
C1864	Maran, Pahang	Telur	*C. gloeosporioides*	555	KT968451
M1567	Telok Mas, Melaka	Melaka Delight	*C. gloeosporioides*	553	KT968456
M1570	Telok Mas, Melaka	Melaka Delight	*C. gloeosporioides*	553	KT968449
M1692	Telok Mas, Melaka	Melaka Delight	*C. gloeosporioides*	554	KT968457
R2258	Kangar, Perlis	Harum manis (MA 128)	*C. gloeosporioides*	556	KT968458
R2275	Chuping, Perlis	Harum manis (MA 128)	*C. gloeosporioides*	566	KT968459

The isolates were classified into *C. gloeosporioides* (15 isolates) and *C. asianum* (18 isolates). All the ITS sequences were deposited (Table 2) in GenBank database with accession number as tabulated in Table 2.

Pathogenicity of *Colletotrichum* isolates obtained from the infected mango

The results from pathogenicity tests showed the disease severity of the isolates was found varied from 1-8 day after inoculation (Table 3). Disease symptoms were observed as a brown to black circular or irregular shape of lesion with the sunken effect on most of the infected fruits (Figure 3). Apparently, a majority of *C. gloeosporioides* species complex isolates started to show the symptom of anthracnose lesion after five days of inoculation and became severe as the duration increases. *Colletotrichum asianum* R2262 appeared as the most pathogenic isolate with DSI of 50% on day 8 after inoculation followed by seven isolates from *C.*

asianum (R2266, R2267, R2279, R2276, B1558, R1808 and R1733) and three isolates of *C. gloeosporioides* (B1508, R2275 and M1567) with all DSI measured by 25%. Besides, there were eight isolates from both species such as *C. asianum* R2278, *C. asianum* R2265, *C. asianum* M1678, *C. asianum* R2271, *C. asianum* R2272, *C. gloeosporioides* B1524, *C. gloeosporioides* C1550 and *C. asianum* R1766) that were considered as least severe by 6.25-18.75% of DSI (Table 3).

Table-3: Disease severity index (DSI) of *Colletotrichum gloeosporioides* species complex

Isolates number	Disease severity index (DSI, %)							
	1	2	3	4	5	6	7	8
Colletotrichum asianum								
B1558	0	0	0	0	0	0	25.00	25.00
M1678	0	0	0	0	0	0	0	12.50
R1733	0	0	0	0	0	0	25.00	25.00
R1766	0	0	0	0	0	12.50	18.75	18.75
R1808	0	0	0	0	0	0	25.00	25.00
R2254	0	0	0	0	0	0	0	0
R2255	0	0	0	0	0	0	0	0
R2257	0	0	0	0	0	0	0	0
R2262	0	8.33	8.33	8.33	8.33	8.33	37.50	50.00
R2263	0	0	0	0	0	0	0	0
R2265	0	0	0	0	0	0	6.25	6.25
R2266	0	0	0	0	0	0	25.00	25.00
R2267	0	0	12.50	12.50	12.50	25.00	25.00	25.00
R2271	0	0	0	0	0	0	12.50	12.50
R2272	0	0	0	0	0	0	12.50	12.50
R2276	0	0	0	0	0	0	25.00	25.00
R2278	0	0	0	0	0	0	6.25	6.25
R2279	0	0	0	0	18.75	18.75	18.75	25.00
Colletotrichum gloeosporioides								
B1477	0	0	0	0	0	0	0	0
B1505	0	0	0	0	0	0	0	0
B1508	0	0	0	0	0	0	0	25.00
B1519	0	0	0	0	0	0	0	0
B1524	0	0	0	0	12.50	12.50	12.50	12.50
B1555	0	0	0	0	0	0	0	0
C1550	0	0	0	0	0	0	18.75	18.75
C1551	0	0	0	0	0	0	0	0
C1553	0	0	0	0	0	0	0	0
C1864	0	0	0	0	0	0	0	0
M1567	0	0	0	0	0	0	25.00	25.00
M1570	0	0	0	0	0	0	0	0
M1692	0	0	0	0	0	0	0	0
R2258	0	0	0	0	0	0	0	0
R2275	0	0	0	0	0	0	25.00	25.00

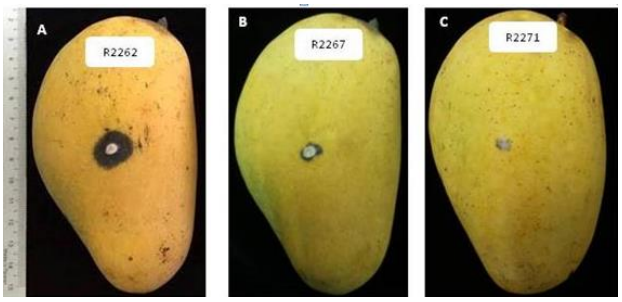

Figure-3: Variation of disease severity of anthracnose on mango.
(A) Black and sunken circular lesion indicated as most severe disease symptom (*C. asianum* R2262); (B)(C) Brown to black lesion with irregular-shape that just extend from colonized PDA plug (*C. asianum* R2267) and least severe (*C. asianum* R2271).

Colletotrichum gloeosporioides species complex isolates started to show the symptom of anthracnose lesion using non-wounded method was after five days of inoculation and becoming severe as the day after inoculation increased. Other studies have shown that inducing of anthracnose lesion on mango was observed in wounded method (Kouame et al., 2010). However, our results corresponded to Agrios (2012) that less effective penetration of fungal isolates were occurred through the natural opening of lenticels. Since the lenticels were opened during growing, these promote the fungal colonizing and increased the severity of mango. The symptoms showed black and sunken circular lesion which was similar with the symptoms of causal agent for anthracnose disease on mango fruits described by previous studies (Arauz, 2000; Weir et al., 2012; Gautam, 2014).
The variation in pathogenicity effect caused by *C. gloeosporioides* species complex in this study was similar to previous study that showed inducing of lesion that was faster in some isolates of similar species compared to the others, which indicates that every isolates obtained have a different capacity in virulence (Kouame et al., 2010; Asma et al., 2018). The high virulent of the isolates might be caused by the capability of the pathogen to produce abundant cell wall degrading enzymes including pectate lyase for breaking down the pectocellulosic wall (Yakoby et al., 2000) which led to the development of anthracnose disease symptoms by softening the mango fruits tissues. In the opposite, the less virulent isolates might be due to lower production of these enzymes. Furthermore, progression of disease severity that increased as day after inoculation increased also coincided with increasing reduction of compounds due to ripening process involved in host defense mechanism (Kouame et al., 2010). The pathogens identified in this study were successfully re-isolated from all the symptomatic mango tissues resulted in fulfilling the Koch's postulates. Meanwhile, control mangoes inoculated with non-colonized PDA plugs remained symptomless until the end of the test.

Conclusion

This study was successfully isolated 33 of fungal isolates of *Colletotrichum* associated with pre harvest anthracnose disease on mango throughout Peninsular Malaysia. The fungal were consisted of *C. asianum* and *C. gloeosporioides*. Pathogenicity of those isolates obtained was determined by carried out the pathogenicity test using non wounded method on healthy mango with the variety of Chokanan. *Colletotrichum gloeosporioides* and *C. asianum* have been confirmed pathogenic towards mango and showed various levels of disease severity of anthracnose. From the total isolates obtained, 19 isolates (57.6%) were confirmed pathogenic by produce at least mild infection with the species of *C. asianum* was recorded the highest percentage of pathogenic isolates. There was also a significant difference in DSI among the isolates.

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Contribution of Authors

Zainudin NAIM: Conceived idea, helped in initiating the experiment, supervise the work and write up of article.
Sattar MM: Conducted experiment, statistical analysis and drafted the result.

Disclaimer: This is to confirm that this manuscript has not been submitted to more than one journal for simultaneous consideration, the manuscript has not been published previously. Consent to submit has been received explicitly from co-author and authors whose

names appear on the manuscript have contributed sufficiently to the scientific work.

Conflict of Interest: None.

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