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Distribution, Isolation and Identification of Leaf spot disease of Papaya (*Carica papaya)* in Peninsular Malaysia

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The disease leaves that fulfilled the criteria as Corynespora Leaf Spot Disease (CLSD) of papaya were collected from twelve places in Semenanjung Malaysia. Disease incidence (DI) and disease severity (DS) recorded and location were marked with Global Positioning System (GPS). Hundred percent of twelves sampling locations was confirmed infected with CLSD. The highest disease severity recorded from Isolate coded as PR2 with 93.3% of DS followed by K1 with 71% of DS. The lowest DS recorded at PH1 with 61.67% of DS. Four fungi were consistently isolated that were *Corynespora* sp., *Culvularia* sp., *Colletetricum* sp., *Rhizoctonia* sp. and it was proven that *Corynespora sp.* was the causal agent of the disease (CLSD) by koch's postulate. Study on the morphology of *Corynespora* sp. colony, mycelium and conidia was done to identify the pathogen. The pathogenic fungi identified using molecular technique and it confirmed as *Corynespora cassiicola*. The amplicons approximately 559bp was amplified using the ITS primers.

Keywords: *Corynespora cassiicola*, Plant Disease, *Carica papaya*, Leaf Spot Disease, ITS Primer.

INTRODUCTION

The identification and diversity analysis play an important role in plant pathogenic fungus studies. It could help to confirm and reveal the inherent variability of the pathogen. Traditionally, the fungal taxonomy and identification of fungi mainly relies on morphological characteristics (Guarro et al., 1999; Glass and Donaldson, 1995; MacLean et al., 1993). However, morphologies are phenotypic characters and do not reveal the genetic homologies among groups of fungi (Glass and Donaldson, 1995; MacLean et al., 1993).

Besides, in morphology, the races or pathotypes of several pathogenic fungus are not easy to distinguish and their identification by cultural characteristics may also be inaccurate because it is based on morphological criteria that are usually depended on cultural conditions, leading to misidentification. Therefore, morphological approach requires expertise, experience and is also time consuming. The emerge of molecular methods with DNA-based tools, especially PCR and DNA sequencing, have supplement traditional taxonomic methods to

study in phylogenetic, systematic, detection, identification of fungi as well as genetic diversity analysis and evolutionary origins in populations of many different fungal genera (Bridge et al., 2005; Carter et al., 2004; Glass and Donaldson, 1995; MacLean et al., 1993).

The information on the distribution, host range and pathogenicity (virulence) of *Corynespora cassiicola* is critical in order to identify the character of this new 'dangerous' pathogen on papaya in Malaysia. Seem this disease is serious in Johor and Terengganu, it is believed that the disease will spread widely to the whole Malaysia as happened to the papaya dieback disease in 2010-2011. Study on the geographical distribution is important to understand the way of dissemination of the pathogen. Hence, identification of the host range and pathogenicity will provide an information of serious threat of the disease to the other commercial crops in the Malaysia. Thus, the objective of this study was to study the distribution, isolate and identify the pathogen(s) responsible for Leaf Spot Disease of papaya.

MATERIALS AND METHODS

Sampling of Leaf Spot Disease of Papaya

Samples were collected from 12 locations. Geographical Positioning System (GPS) [Model GARMIN GPSmaps 62s, USA] was used to mark the distribution of the suspected diseases papaya all over Peninsular Malaysia. The location (area) of the infection recorded base on regular symptom of Corynespora Leaf Spot Disease (CLSD) present on the leaf of papaya. The areas were marked by GPS reading.

Disease Incidence and Disease Severity

To evaluate the percentages of the infection, Disease incidence (DI) was assessed based on the number of samples affected from the total number of samples examined, expressed as the percentage of diseased plants (James, 1974; Kranz, 1988; Lorbeer, 1978).

$$\text{DI (\%)} = \frac{\text{(Number of samples infected)}}{\text{(Number of all samples)}} \times 100$$

To evaluate the severe of pathogen infection, Disease severity (DS) was based on the area of plant tissue showing symptoms of the disease (Kranz, 1988). Data were collected based on the existence on the leaf of the plants and divided to the number of all plant in the area with minimum 5 number of plant as one samples.

Isolation of Disease Pathogen

For this study, isolation of the associated organism with the infected leaves of papaya were done from North, East, South and West regions of Peninsular Malaysia. Samples of infected leaves were brought to the laboratory following the isolation process. Isolation was done using standard methods with PDA as media (Hailmi et al., 2013; Kadir et al., 2011; Ng et al., 2012). For this purpose, the samples were cut to small pieces (1 to 2 cm), surface sterilized in 1% NaOCl, and plated onto PDA in petri dishes and incubated at 30^{o}C.

Pure isolates were obtained by subculturing and single spore methods. For this purpose, the suspension of spores from 10 days old culture was prepared and diluted to about 100 spores/ml in sterile water. A quantity of 0.25 ml diluted spore suspension was spread onto an agar petri dish. A well-separated single spore was selected and marked under stereo microscope. The marked spores were picked up using a flamed needle, placed separately onto PDA petri dish (5 spores/plate) and incubated at 25 °C for 24 hours. The well grow colony was then selected and sub-cultured onto new PDA petri dish.

Morphological Characteristics

Colony morphology

Five mm mycelial plug taken from the actively growing at the end of colony of 5-day old culture of the fungal isolates on PDA. Cultures were incubated in the dark in incubator at temperature (28 +- 2°C). The experiment was in five replicate for each isolate and arranged using completely randomized design (CRD). Colony's characteristics such as shape, color of colony and structure were recorded. Measurement of colony diameter was recorded daily by taking the average length of two diameters at right angles for a period of seven days using a ruler. Data were subjected to analysis of variance (ANOVA) using SAS software version 8.01.

Conidial morphology

Conidia of the isolates were produced using Half-Strength PDA on the aluminum trays. The leaflets were inoculated by pipetting 20 µl drops of a spore suspension (400 spores/ml) at 10 spots/leaflet. The inoculated leaflets were placed under fluorescent light in air-conditioned room (25^{o}C) from 7 to 10 days. The produced spores were collected and stained using lactophenol blue

(LCB) and fixed onto glass slides for observation using microscope. The conidial morphology and dimensions of the isolates were photographed by using LEICA DM500 B Microscope.

Molecular characterization

Fungal mycelium preparation

Pure cultures of the isolates were sub-cultured by placing five mycelial plugs taken from the actively growing margin (end of colony) of 5-day old culture into Erlenmeyer flasks containing 200 mL Difco™ Potato Dextrose Broth (24 g/L). The flasks were shaken at 120 rpm using an orbital shaker at room temperature (28 +- 2°C) for 12 days. The mycelia were filtered through a layer of cheese cloth, and washed with distilled water and immediately stored in a deep freezer at -80^{o}C.

Fungal DNA extraction

DNA extraction was performed using modified CTAB method as described by c. For this purpose, a quantity of 250 mg frozen mycelia was ground in liquid nitrogen using pre-chilled mortar and pestle. A volume of 5 mL preheated (65^{o}C) CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA pH 8.0; 100 mM Tris-HCl pH 8.0 and 0.3% 2-mercaptoethanol) was added into the ground tissue. The suspension was incubated at 65^{o}C for 90 minutes with occasional mixing. The mixture was cooled to room temperature and mixed well with an equal volume of chloroform:isoamyl alcohol (24:1) and emulsified. After centrifuged at 10,000 rpm for 10 minutes at room temperature the aqueous phase of the mixture was transferred to another tube. RNase A solution (25 µl, 20 µg/mL) was added into the tube, mixed well by inversion and incubate at 37^{o}C for 30 minutes. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed for 10 minutes by inversion. The mixture was centrifuged at 10,000rpm for 10 minutes at room temperature and the aqueous phase was transferred to another tube. The DNA was precipitated by adding 0.6 volume of cold isopropanol, then slowly swirling the tube and incubated at -20^{o}C for 1 hour. After centrifuged at 10,000 rpm for 10 minutes at 4oC, the supernatant was decanted and the DNA pellet in the tube was washed twice with ethanol 70% and air-dried at room temperature until there was no trace of ethanol. The DNA pellet was then dissolved in 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0).

Eppendorf BioPhotometer were used to measure the purity and the concentration of extracted DNAs. The integrity of the extracted DNA was checked by gel electrophoresis with 1.2 % agarose gel under 1x TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) at 70 V for 45 minutes at room temperature. The molecular weight of the extracted genomic DNA was estimated by comparison with a 1kb DNA Ladder (0.25 - 10 kb) marker (Promega). The DNA were then stored in the refrigerator at 4^{o}C and used for PCR reaction. A NanoDrop ND-1,000 spectrophotometer (LMS Co., Ltd., Tokio, Japan) was used to check the quality and concentration of Genomic DNA.

PCR amplification

The amplification of 28S large subunit ribosomal DNA gene was performed in a thermocycler (DNA Engine® Peltier Thermal Cycler PTC-200, MJ research, USA) with initial denaturation at 94^{o}C for 5 minutes, followed by 35 cycles of denaturation at 94oC for 30 seconds, annealing at 55^{o}C for 30s minute, extension at 72^{o}C for 30s and the final extension at 72^{o}C for 2 minutes. The primer pairs used to amplify the gene were as followed; Forward LROR 5'-ACCCGCTGAACTTAAGC-3' and LR7Reverse 5'-TACTACCACCAACATCT-3'. The amplified products were size-separated in 1.2 % agarose gel under 1x TAE buffer (40 mM Tris, 20 mM Acetic acid and 1 mM EDTA) at 70 V for 45 minutes at room temperature. Gels were stained with GelRed (Biothium, USA), visualized under UV light and photographed using a gel documentation system (GeneSnap, Ver 6.03, Syngene Laboratories). The sizes of amplified DNA fragments were estimated using GeneTools (Ver 3.00.13, Syngene Laboratories) by comparison with a 2-Log DNA Ladder (0.1-10 kb) marker (BioLabs).

DNA sequencing and analysis

Amplified products were purified using the PCR Mega-Quick SpinTM product purification kit (Intron, Biotechnology, Korea). Briefly, 100 µl of binding buffer was added into a 1.5 ml microcentrifuge tube containing the PCR-amplified product and mixed well. Meanwhile, a spin column was placed in a 2 ml collection tube. The suspension was transferred into the spin column and centrifuged for 1 min at 10000rpm at room temperature. After centrifugation, the flow-through was discarded and 500 µl of washing buffer (containing ethanol) was added into the column

and centrifuged for 1 min at 10000rpm at room temperature. The column was washed again with washing buffer and the flow-though was discarded. In order to remove the residual buffer, centrifugation was repeated again for 1 min. The purified amplified products were sent to a commercial company (1st Base Pte Ltd, Malaysia) for sequencing. Results of the DNA sequencing were then compared to the GenBank database of the National Centre for Biotechnology Information (NCBI) using the Nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTN) software version 2.2.14 for identification.

RESULTS

Sampling

Sampling of pathogenic fungal isolates was done at 12 places from 9 states as representatives for the main papaya growing regions in Semenanjung Malaysia as listed in Table 1. The sampling areas were marked with GPS (Garmin GPSmaps 60s) reading on Longitudes.

Disease Incidence and Disease Severity of Corynespora Leaf Spot Disease in Malaysia

During sampling, data on disease incidence and disease severity was recorded. The results indicated that all examined papaya trees had 100% disease incidence (Table 2). Meanwhile, PR2 showed the highest value of disease severity with 93.30% followed by N1 with 80.71%, and the PH1 showed the lowest disease severity with 61.67% (Table 2).

Table 1: Selected locations of sample collection in Peninsular Malaysia.

	Sample No.	Area	Longitudes
North Zone	KD1	TKPM, Gajah Mati, Pokok Sena, KEDAH	N 06°09'56.6" E 100°32'58.2"
	PR2	Sungai Jambu, Selama, PERAK	N 05°11'20.4" E 100°41'18.4"
	PP1	Sungai Bakau, Nibong Tebal, PULAU PINANG	N 05°07'58.4" E 100°24'40.1"
South Zone	M1	Paya Lebar, Ramuan Cina Besar, MELAKA	N 02°24'30.9" E 102°03'45.1"
	M2	Pengkalan Balak, MELAKA	N 02°20'09.81 E 102°03'58.37
	N1	Rantau, NEGERI SEMBILAN	N 2°34'11.05" E 01°55'10.66"
East Zone	T1	Gong Badak, Kuala Terengganu, TERENGGANU	N 5°24'09.78" E 03°05'00.05"
	T2	Gong Medang, Besut, TERENGGANU	N 5°45'24.07" E 102°34'07.60"
	PH	Kuala Lipis, PAHANG	N 4°11'01.58" E 102°02'41.78"
West Zone	S1	UPM, Serdang, SELANGOR	N 2°58'50.01" E 101°42'46.26"
	S2	Ijok, Selangor	-
	KL	Cheras Perdana, KUALA LUMPUR	N 3°03'22.91" E 101°44'22.12"

*North Zone (Perak, Kedah, Pulau Pinang), Southern Zone (Johor, Melaka, Negeri Sembilan), East Zone (Terengganu, Pahang), West Zone (Selangor, Kuala Lumpur)

Table 2: Disease Incidence and Disease Severity of 12 samples collected from Peninsular Malaysia.

Location	Sample No.	Disease Incidence (%)	Disease Severity (%)
North Zone	K1	100	71.67
	PR2	100	93.30
	PP1	100	66.67
Southern Zone	M1	100	66.67
	M2	100	66.00
	N1	100	80.71
East Coast Zone	T1	100	66.67
	T2	100	62.00
	PH 1	100	61.67
West Coast Zone	S1	100	68.00
	S2	100	68.75
	KL	100	63.00

*North Zone (Perak, Kedah, Pulau Pinang), Southern Zone (Johor, Melaka, Negeri Sembilan), East Zone (Terengganu, Pahang), West Zone (Selangor, Kuala Lumpur)

Isolation

A total of 12 fungal isolates was collected from 12 places (Table 3). All isolates were recovered from the infected leaves of papaya trees showing Corynespora Leaf Spot.

Five common fungi were isolated from all 12 samples collected. The fungi were initially identified as *Corynespora* sp., *Culvularia* sp., *Colletrotricum* sp., and *Ryzocthonia* sp. (Table 3, Figure 3). *Corynespora* sp. was consistently present at all 12 papaya planted areas. *Coletrotricum* sp. was isolated from four samples of K1, N1, T2 and S1. *Rhizoctonia* sp. was only isolated from N1. Meanwhile, *Culvularia* sp. was obtained from six samples of K1, PP1, M2, T2, PH and KL.

Table 3: Several fungal isolates obtained from diseased leaf samples collected from North zone, Southern zone, East zone and West zone of peninsular Mapaysia.

SAMPLE	Code	*Corynespora* sp.	*Culvularia* sp.	*Colletetricum* sp.	*Rhizoctonia* sp.
North Zone	K1	+	+	+	-
	PR2	+	-	-	-
	PP1	+	+	-	-
Southern Zone	M1	+	-	-	-
	M2	+	+	-	-
	N1	+	-	+	+
East Zone	T1	+	-	-	-
	T2	+	+	+	-
	PH	+	+	-	-
West Zone	S1	+	-	+	-
	S2	+	-	-	-
	KL	+	+	-	-

* (+) = Yes, (-) = Absence, 0 = Present of *C. cassiicola* with Other Fungi

*North Zone (Perak, Kedah, Pulau Pinang), Southern Zone (Johor, Melaka, Negeri Sembilan), East Zone (Terengganu, Pahang), West Zone (Selangor, Kuala Lumpur)

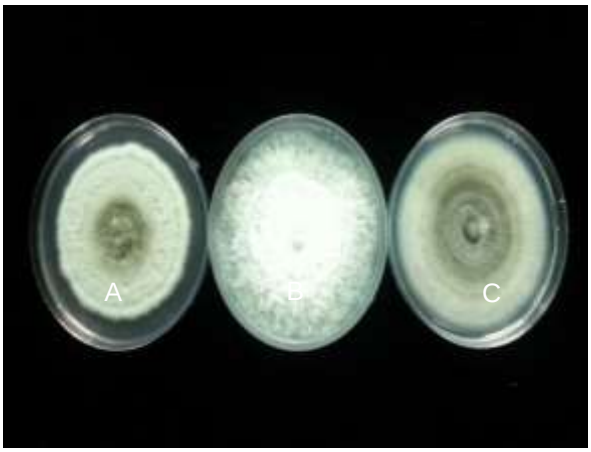


Figure 1: Several fungi isolated from diseased leaf sampes (Corynespora leaf spot); (A) *Corynespora* sp., (B) *Ryzoctonia* sp., and (C) *Colletotricum* sp.

Morphological characteristics

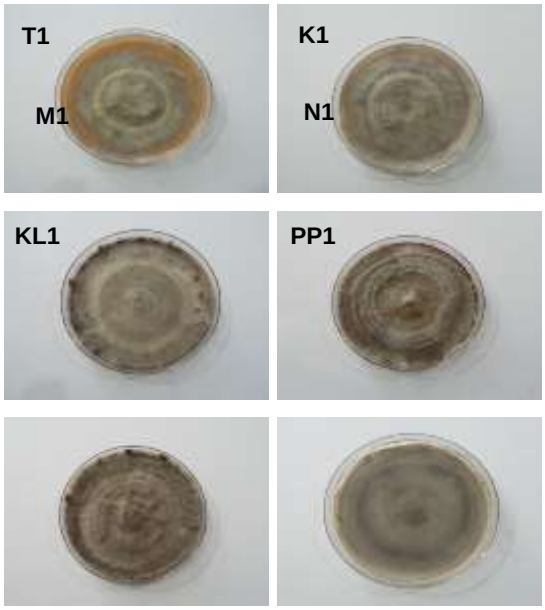
Colony morphology

Twelve fungal isolates obtained from all 12 places had been observed closely in the lab focusing on the character of the formation of the colony and the colour at 25ºC after 7 days (Table 4, Figure 2). The colonies were light grey, grey and dark grey on the top and black at the bottom of PDA plates. Ring shape were observed on the top of the colonies. The orange colour around the colonies were seen on the media during the growth of the isolates on PDA.

Table 4: Colony colour description of fungal isolates collected from different location in Peninsular Malaysia.

Sample Loccations	Colour		
	Code	Upsite	Bottom
North Zone	K1	Grey	Black
	PR2	Dark Grey	Black
	PP1	Grey	Black
Southern Zone	M1	Grey	Black
	M2	Grey	Black
	N1	Grey	Black
East Zone	T1	Light Grey	Black
	T2	Dark Grey	Black
	PH1	Grey	Black
West Zone	S1	Dark Grey	Black
	S2	Dark Grey	Black
	KL1	Light Grey	Black
	KL	Grey	Black

*North Zone (Perak, Kedah, Pulau Pinang), Southern Zone (Johor, Melaka, Negeri Sembilan), East Zone (Terengganu, Pahang), West Zone (Selangor, Kuala Lumpur)



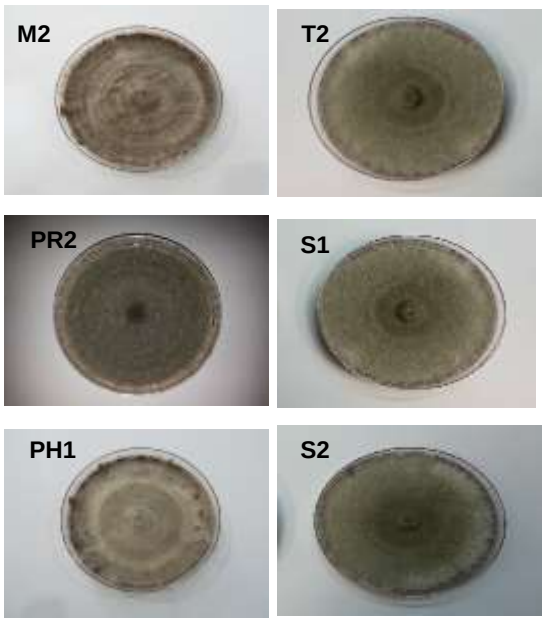


Figure 2: Colony morphology of 12 fungal isolates obtained from 12 different places in peninsular of Malaysia. K1 (Kedah), PR2 (Perak), PP1 (Pulau Pinang), M1 (Melaka), M2 (Melaka), N1 (Negeri Sembilan), T1 (Terengganu), T2 (Terengganu), PH (Pahang), S1 (Selangor), S2 (Selangor), KL (Kuala Lumpur).

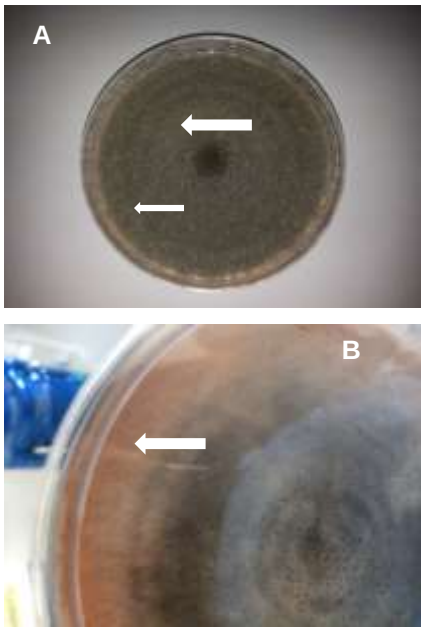


Figure 3: Colony of *Corynespora* sp. colony on day 11 of incubation in 25ºC (A) on PDA media. Colony of *Corynespora sp.* on PDA with orange colour of PDA (B). The hypha of the *Corynespora* sp. viewed under light microscope with 40x magnification (C).

Conidial morphology

Fifty conidia of each isolate were randomly observed under light microscope. The conidia were septate from 4 to 19 septate in range. Conidia were range from 7 to 25 µm in width and were range from 40 to 280 µm in length. As shown in Figure 7, the shape of a conidium was slightly curved (Figure 7A) normally in chained together with a range of 3 to 4 conidia in range for each chain (Figure 4). Hypha were thicker towards the conidiophore compared to the hypha at the others parts.

Observation of conidia germination of fungal isolates has been done. The germ tube was observed after 2 hour in sterile distil water or onto the water agar media viewed under light microscope with 40x magnification (Figure 5). Conidia could germinate from both end septate of conidia and at side of conidia on septate number 3, 4 and 5 (Figure 5) counted from the conidiophore end attached of conidia.

Based on morphological characteristics examined in this study, all 12 obtained isolates were similar with original description of *Corynespora cassiicola* (Ellis and Holiday 1971) as shown in Table 5.

Table 5: Comparison on the size of conidia of isolated *Corynespora* sp. as described by Ellis and Holiday (1971).

Isolate	Width Range (µm)	Length Range (µm)	Pseudosepta
***C. cassiicola*[a]**	7 - 25	40 - 280	4-19
***C. cassiicola*[b]**	8 - 22	40 - 220	4-17
***C. cassiicola*[c]**	5.14 – 12.85	59.11-174.76	-

*[a] Isolated from diseased leaves
*[b] Ellis, M. B., and Holiday, P. (1971)
*[c] Neni Kartini (1995)

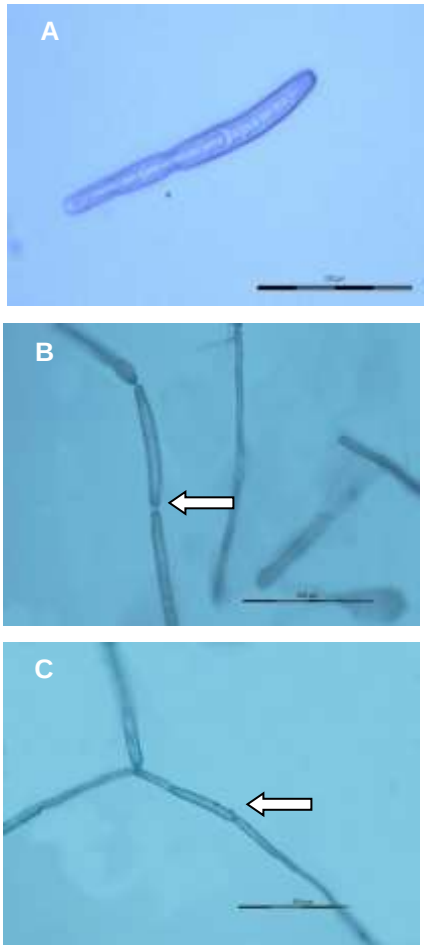


Figure 4: Conidia and hypha of *Corynespora* sp. view under LEICA DM500 B microscope under 40x magnification.

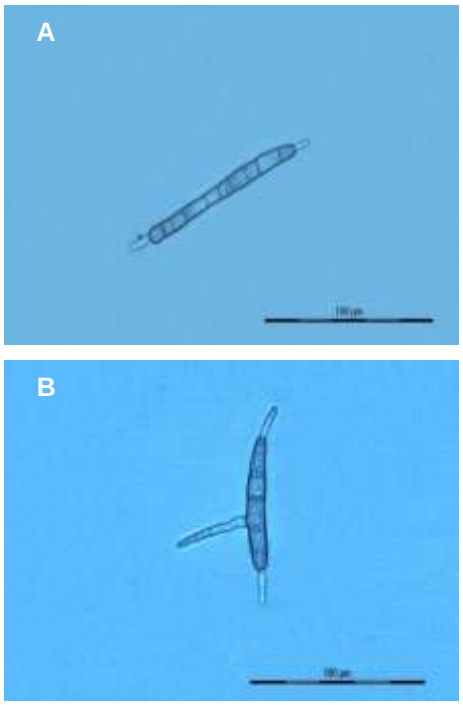


Figure 5: Germination of *Corynespora* sp. view under LEICA DM500 B Microscope with 40x magnification.

Pathogenicity

Pathogenicity test of *C. cassiicola* isolates has been done to confirm the causal agent of leaf spot disease of papaya. The symptom of disease shows by *C. cassiicola* in the experiment was very much similar to the symptom appear in the sampling fields. The spot was started with small yellow spot (Figure 5) at day four after inoculation, then enlarges with grey colour in the center of the spot surround with brown and yellow hallow around the spot. Center of the spot then becomes dry, decomposted and formed a holes and the yellow hallo surround the spot will enlarged. At the severe stage of infection, the whole leaf become yellow and most of the spot will become a hole in centre. The process has been repeated three times and confirmed that the *C. cassiicola* was the causal agent of papaya leave spot disease. Based on the result, *C. cassiicola* labelled as PR2, originated from Sungai Jambu, Selama, Perak was selected for further study.

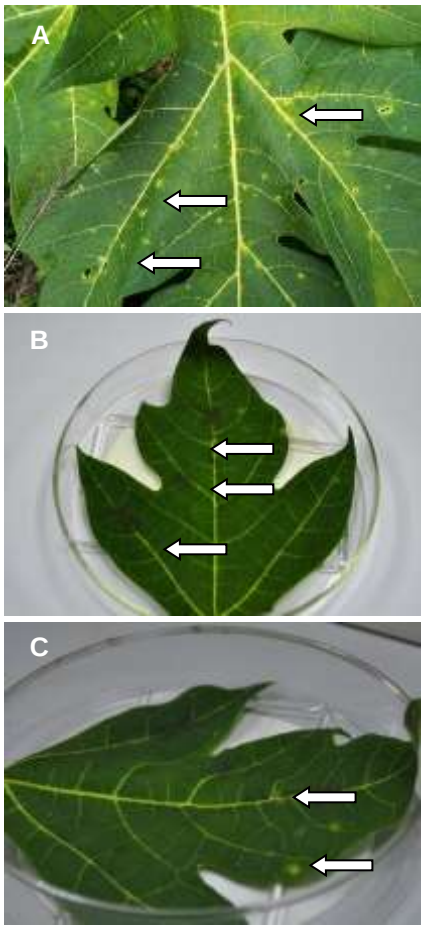


Figure 6: Disease symptom of *C. cassiicola* on the leaf of papaya as seen in the field (A) and as the result of Koch's Postulate conducted in the laboratory (B) and (C) after 3 days of inoculation.

Molecular identification

The ITS region (including partial regions of 18S rDNA, ITS1, 5.8S, ITS2 and 28S rDNA) from the representative isolate PR2 were successfully amplified and sequenced with 559 bp. BLASTn alignment was conducted to identify and analyze homologous sequences with those of *Corynespora* species deposited in the GenBank. The consensus sequence of PR2 showed 99% homology with *C. cassiicola* isolates available in the GenBank under Accession Nos. MG976665, MG976664, and MG976663. The new sequence obtained in this study was deposited in the GenBank. The result confirmed the morphological identification of the pathogenic fungus as *C. cassiicola*.

DISCUSSION

Corynespora cassiicola is one of the most important destructive pathogens of papaya. In Malaysia, *C. cassiicola* is renowned as the main pathogen in rubber industry (Nghia et al., 2008), and it was consider main pathogen on papaya for a long time ago at 1980. Therefore, a comprehensive study on the identification and distribution of *C. cassiicola* as an important pathogenic fungus on papaya is necessary in Malaysia.

Many studies have reported *C. cassiicola* on various host plants around the world indicating that *C. cassiicola* has a wide host range. For instance, *C. cassiicola* has been reported on *Codiaeum variegatum* in Sri Langka (Jayasuriya & Thennakoon, 2007), okra in Bangladesh (Ahmed et al., 2013), *Pueraria phaseoloides* in Brazil (Lisboa et al., 2016), cucumber in Japan (Miyamoto et al., 2010), and papaya in Florida, USA (Conover, 1978).

In this study, 12 fungal isolates were obtained from all 12 samples collected from papaya planted areas from 10 states as representatives for main papaya growing regions in Sememananjung Malaysia. The disease incidence was recorded 100% for all 12 papaya planted areas. The results of morphological characteristics indicated that the obtained fungal isolates are similar to the original description of *C. cassiicola* (Ellis and Holliday, 1971). In this respect, Conidiophore emerged through leaf epidermis, slightly or conspicuously at apex, simple, single, determinate or in turfs, prolifering terminally through scar of previous conidium (Barnett and Hunter, 1998). Conidia terminal, single or sometimes in short chains (Barnett and Hunter, 1998).

Pathogenicity assay revealed that all *C. cassiicola* isolates were pathogenic on the papaya leaves in the laboratory similar to those observed in the infected fields, days after inoculation. Re-isolation of the fungus with the same morphological characters with the original isolates was confirmed by Koch's postulates. Therefore, this results confirmed that *C. cassiicola* isolates were the causal agents of leaf spot on papaya trees. The results also showed a high level of virulence variability among the isolates. Among the isolates, PR2 indicated the highest virulence variability.

Molecular markers have been widely adopted to determine the genetic characteristics of fungi, plants and animals. Molecular techniques based on detection, such as sequencing of different DNA genes have been used as alternatives to morphological identification of *C. cassiicola* (Oktavia and Dinarti, 2017; Xu et al., 2016). ITS-rDNA is the sequence with a repeat unit of the ribosomal genes that is frequently used for genome clustering of the fungi (Nilsson et al., 2009). This region can show the variation between the fungal species by the difference in length of sequences due to the insertions or deletions or as a result of nucleotide substitutions without altering the number of bases (Katoch et al., 2015). Previous reports indicated that ITS-rDNA a very useful region for the identification of *C. cassiicola* (Oktavia and Dinarti, 2017). In this study, the representative isolate PR2 with the highest virulence variability was characterized by molecular identity at the ITS-rDNA region. The result BLASTn alignment with high similarity indicated that the isolate belonged to *C. cassiicola;* and therefore, the morphological identification was confirmed.

CONCLUSION

Disease incidence of leaf spot disease in all 12 papaya production areas were recorded as 100%. In this study, 12 fungal isolates were obtained from 12 main papaya production areas in Malaysia in 2013. According to morphological and molecular characteristics, all isolates were identified as *C. cassiicola.* Pathogenicity test also indicated that *C. cassiicola* is response for the leaf spot in all areas.

As the Koch's postulate confirmed the *C. cassiicola* was the causal agent of leaf spot disease. All four regions of Peninsular Malaysia where the samples were collected from infested papaya which covers leaves spot disease with 100% disease incidence. Not to be confused, the 100% incidence doesn't mean that an infecton was all at serious level. Some of the infection was not harms the plant at all. The harmfull effect of the pathogen (*E. cassiicola*) infection is measured using the disease severity scale (Baharon et al., 2014; Hailmi et al., 2011). Sample PR2 from Sungai Jambu, Selama, and Perak was the highest disease severity with 93.30% and PH1 was the lowest with 61.67%.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

MHS design and performed the experiment. Others author (KJ, KS, MTM, NAB, MMK and SM) rad and approved the final version.

REFERENCES

Ahmed FA, Alam N, & Khair A, 2013. Incidence and biology of Corynespora cassiicola (Berk. & Curt.) Wei. disease of okra in Bangladesh. Bangladesh Journal of Botany, 42(2): 265–272.

Baharon MN, Kadir J, Juraimi AS, Man A, Sajili MH, & Abu Bakar NK, 2014. Exserohilum monoceras: Potensinya untuk mengawal rumput miang (Leptochloa chinensis) dalam tanaman padi tabur terus. Jurnal Teknologi, 70(6): 53-56

Barnett HL, & Hunter BB, 1998. Illustrated Genera of Imperfect Fungi. APS Press, 38(4), 480.

Bridge ES, Jones AW, & Baker AJ, 2005. A phylogenetic framework for the terns (Sternini) inferred from mtDNA sequences: Implications for taxonomy and plumage evolution. Molecular Phylogenetics and Evolution, 35(2): 459–469.

Carter D, Nai T, Marra R, & Vera R, 2004. The development of genetic markers from fungal genome initiatives. Applied Mycology and Biotechnology, 4: 1–27.

Conover RA, 1978. Corynespora Leaf Spot , A Disease Of (Fig- I) -. Proc. Fla. State Hort. Soc., 91: 184–185.

Déon M, Scomparin A, Tixier A, Mattos CRR, Leroy T, Seguin M, Pujade-Renaud V, 2012. First characterization of endophytic Corynespora cassiicola isolates with variant cassiicolin genes recovered from rubber trees in Brazil. Fungal Diversity, 54: 87–99.

Ellis MB, & Holliday P, 1971. Corynespora cassiicola [target spot]. C.M.I. Descriptions of Pathogenic Fungi and Bacteria.

Glass & Donaldson, 1995. Development of premier sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Applied and Environmental Microbiology, 61: 1323–1330.

Guarro J, GenéJ J, & Stchigel AM, 1999. Developments in fungal taxonomy. Clinical Microbiology Reviews, 12(3): 454–500.

Hailmi MS, Jugah K, Abdullah TA, Suhaili Z, Mohamad NM, Baharon MN, Mohamed S, 2011. Potential of Exserohilum monoceras as Bioherbicide for Controlling Echinochloa crus-galli (Rumput Sambau). Journal Of Agrobiotechnology, 2(0): 1–15.

Hailmi MS, Jugah K, Ng SC, & Rahim AA, 2013. Effect of Inoculum Form of on Disease Development Exserohilum longirostratum on Disease Development. Journal Of Agrobiotechnology, 4(0): 43–56.

James W, 1974. Assessment of plant diseases and losses. Annual Reuieu-T of Phgtopathotogg, 12: 27–48.

Jayasuriya KE, & Thennakoon BI, 2007. Short Communication First Report of Corynespora Cassiicola on Codiaeum Variegatum (Croton) in Sri Lanka. Plant Pathology, 36(2): 138–141.

Kadir J, Hailmi MS, Charuddatan R, & Rosskopf, EN, 2011. Effects of Culture Age on Conidi Production, Viability and Pathogenicity of Dactylaria higginsii Kadir. Journal Of Agrobiotechnology, 2(0): 17–23.

Katoch A, Prabhakar CS, and Sharma PN, 2015. Metageographic population analysis of Colletotrichum truncatum associated with chili fruit rot and other hosts using ITS region nucleotide sequences. J. Plant Biochem. Biotechnol. 25: 64-72.

Kranz J, 1988. Measuring plant disease. In J. Kranz & J. Rotem (Eds.), Expertmental" techni4ues in plant disease epidemioLog. . Springer-Verlag, Berlin.

Lisboa WS, Macedo DM, Silva M, & Barreto RW, 2016. First report of Corynespora cassiicola on Pueraria phaseoloides (tropical kudzu). Australasian Plant Disease Notes. https://doi.org/10.1007/s13314-016-0203-8

Lorbeer JW, 1978. Plant Disease: An Advanced Treatise. Volume 1: How Disease is Managed . James G. Horsfall , Ellis B. Cowling. The Quarterly Review of Biology, 53(4): 462–463.

MacLean D, Braithwaite K, Manners J, & Irwing J, 1993. How do we identify and classify fungal pathogens in the era of DNA analysis? Advances in Plant Pathology, 10: 207–244.

Miyamoto T, Ishii H, Stammler G, Koch A, Ogawara, T, Tomita, Y, & Kobori, S, 2010. Distribution and molecular characterization of Corynespora cassiicola isolates resistant to boscalid. Plant Pathology, 59(5): 873–881.

Ng SC, Kadir J, & Hailmi MS, 2012. Histological study of the interaction between Exserohilum Longirostratum, barnyard grass, and rice var. Mr219. Pertanika Journal of Tropical

Agricultural Science, 35(SUPPL.).

Nghia NA, Kadir J, Sunderasan E, Puad Abdullah M, Malik A, & Napis S, 2008. Morphological and Inter Simple Sequence Repeat (ISSR) markers analyses of Corynespora cassiicola isolates from rubber plantations in Malaysia. Mycopathologia, 166(4): 189–201.

Nilsson R, Ryberg M, Abarenkov K, Sjokvist E, and Kristiansson K, 2009. The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. FEMS Microbiol. Lett. 296: 97-101.

Oktavia Fetrina, & Diny D, 2017. Pathogenicity and rDNA-ITS sequence analysis of the Corynespora cassiicola isolates from rubber plantations in Indonesia. Emirates Journal of Food and Agriculture: 872-883.

Xu J, Qi X, Zheng X, Cui Y, Chang X, and Gong, G, 2016. First report of Corynespora leaf spot on sweet potato caused by Corynespora cassiicola in China. Plant Disease, 100(10): 2163-2163.