

Microfungi Associated with Leaf Spot of Cocoa in Pahang, Malaysia

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ABSTRACT

Crop diseases caused by fungal infections have significantly impacted cocoa production, leading to substantial economic losses due to ineffective disease management strategies. Leaf spot disease, in particular, threatens cocoa yield and quality, necessitating accurate identification of its causal agents for effective control measures. This study aimed to isolate, identify, and assess the pathogenicity of fungal species associated with cocoa leaf spot disease. A total of 45 microfungi isolates were obtained from infected cocoa leaves and identified to the genus level, including *Aspergillus*, *Clonostachys*, *Colletotrichum*, *Curvularia*, *Diaporthe*, *Fusarium*, *Lasiodiplodia*, and *Nigrospora* species. Pathogenicity tests revealed that 12 isolates induced lesions on cocoa leaves, confirming their pathogenic potential. Molecular identification using internal transcribed spacer (ITS) sequence analysis verified the species identity of the pathogenic isolates, with *Lasiodiplodia theobromae* (B3418) exhibiting the highest virulence, producing lesions of 3.17 ± 0.76 mm. These findings highlight the diverse pathogenic fungi associated with cocoa leaf spot disease. This study provides valuable insights into fungal pathogenicity and can contribute to the development of effective disease management strategies, which can reduce fungal infections and improve cocoa productivity.

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INTRODUCTION

Cocoa (*Theobroma cacao* L.) is commonly planted due to its natural range and contribute to the agricultural sector (Norhayati *et al.*, 2016). In

Malaysia, cocoa production reaching its peak in the 1990s before experiencing a decline due to environmental and economic challenges (Arshad and Ibragimov, 2015; Omar *et al.*, 2018). One of the

key factors affecting cocoa productivity is the prevalence of diseases, which can cause significant yield losses. Despite the decline, cocoa remains a valuable crop, with production reaching 360 tonnes across 5,995 hectares of cultivated land in 2021 (Ministry of Plantation Industries and Commodities, 2021).

However, the sustainability of cocoa cultivation is threatened by various diseases, which can significantly reduce yields and affect the overall industry. Among the major diseases affecting cocoa in Malaysia, Vascular Streak Dieback (VSD), caused by *Oncobasidium theobromae*, has been well-documented for its severe impact on seedlings and mature trees (McMahon & Purwantara, 2016, p. 307). However, leaf spot disease, which affects the photosynthetic capacity and overall health of cocoa plants, has received comparatively less attention. While various studies have explored cocoa diseases, there is limited research specifically addressing the fungal pathogens responsible for leaf spot disease, creating a knowledge gap in pathogen identification and management strategies.

Environmental factors, such as climate variability and plantation conditions, can influence fungal adaptation and disease severity, potentially leading to the emergence of new pathogenic strains. Without proper identification and monitoring, these pathogens may spread and causing disease outbreaks. Accurate identification of causal agents is crucial for effective disease management. Various studies have been previously conducted related to cocoa diseases. However, there is limited documentation on cocoa pods and leaves disease, especially leaf spot disease. Moreover, the late disease management also has caused losses in cocoa productivity because of affected plant organs, specifically pods and leaves.

Given the improved interest in cocoa cultivation in Malaysia and its potential for economic development, understanding the fungal diversity associated with leaf spot disease is essential. This

study aims to fill the existing knowledge gap by characterizing the microfungi associated with cocoa leaf spot disease, assessing their pathogenicity, and confirming the identity of pathogenic isolates using ITS sequence analysis. The findings from this study will contribute to improve disease management strategies, which can enhance cocoa productivity and sustainability.

MATERIALS AND METHODS

Sampling and Fungal Isolation

Cocoa leaves that showed the symptoms of fungal infection were collected at Cocoa Research and Development Centre (CRDC) Jengka, Pahang, located at 3°36'53.3052"N, 102°30'37.9548"E in March 2022. The cocoa leaves were collected randomly in the field and conveniently around the area based on considering the variability of cocoa trees. The leaf sample that was collected may show some typical symptoms such as chlorosis and leaf spots. A total of 30 leaf samples were collected, with five samples taken from each variety (PBC123, BR25, PBC140, KKM1, KKM5, and KKM22). All the infected samples were kept in a paper bag and labelled with sample number, date, and variety. The collected samples were carried to the laboratory to perform the fungal isolation.

The potential spots were chosen on the leaves, and it was cut into small pieces which were 2 mm². The small pieces of tissues were surface disinfected and cultured on Potato Dextrose Agar (PDA) plates. The plates were incubated at room temperature (28 ± 2°C) for 5-7 days before purification (Alsohaili and Bani-Hasan, 2018).

The fungal cultures were subcultured onto Water Agar (WA) plates for fungal purification using the streaking technique (Elechi et al., 2022). The culture plates were incubated for 24 hours. The hyphal tip of the isolates was transferred onto the new PDA plates to obtain the pure isolates (Yao et al., 2022).

Morphological Characterization

The PDA plates of pure isolates were incubated for 5 to 7 days to allow the mycelial growth and conidia production. All isolates were observed based on the macroscopic and microscopic characteristics. In macroscopic characteristics, the isolates were observed on their colony features and pigmentation. The colony features include the colony form, elevation, margin, and surface (Gaddeyya et al., 2012). In microscopic characteristics, the isolates were observed under the glass slides using a compound microscope (Olympus CX31) under 100x magnification. The conidia shape, length, and width were measured for each of the isolates. As the conidia may be colorless, the methylene blue was used in slide observation to aid in identification and observation of the conidia. The shape and size of the conidia were photographed and measured using Dino-Eye Edge Digital Eye-Piece Camera model AM7025X.

Pathogenicity test

Healthy seedlings of the 3-month-old variety MCBC1 cocoa grown were purchased from CRDC Jengka, Pahang. The 7-day-old fungal culture on PDA was used for the pathogenicity test by inoculating fungal mycelial plugs (5 mm in size) following the procedure by Huda-Shakirah et al. (2022). All leaves were surface sterilized and three wound points were produced on the leaves using sterilized needles (Huda-Shakirah et al., 2022). The mycelial plugs were inoculated upside down onto the three wounded points. All treatments and controls were completed in six replicates for each. The control set was inoculated with a PDA disk. All cocoa seedlings were then incubated for 24 hours to promote fungal growth. The test was conducted for 21 days under controlled conditions, in the plant house at the Department of Biology, Universiti Putra Malaysia.

Lesions that developed on the inoculated cocoa leaves were measured and recorded. The fungi from the symptomatic inoculated leaves were reisolated and identified to fulfil Koch's postulates.

The means of lesion length data for each replicate were tabulated. The statistical analysis of the pathogenicity test data was performed by using IBM SPSS version 26 software for Windows where it involved one-way ANOVA. The mean value then was compared by using Tukey's test.

Species Identification of the Pathogenic Isolates based on ITS Sequence Analysis

To accurately determine species of all pathogenic isolates, molecular identification was performed using the internal transcribed spacer (ITS) region, a widely used DNA barcode for fungi due to its high variability among species (White et al., 1990).

The gDNA of the pathogenic isolates were extracted using the UltraClean® microbial DNA isolation kit (MO-BIO, Carlsbad, CA, USA) following the manufacturer's guidelines. The ITS region of the isolates was amplified using polymerase chain reaction (PCR) using master mix (Promega, Madison, WI, USA) according to the manufacturer's guidelines. To perform the ITS rDNA gene amplification, two pair of primers where ITS1 which 5' TCC-GTA-GGT-GAA-CCT-GCG-G 3' and ITS4 which 5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3' was applied (White et al., 1990). About 4 µl of 5x GoTaq buffer, 2.0 µl of 2 mM dNTPs and MgCl₂, 1 µl of 10 mM primers ITS1 and ITS4, 1 µl of 5 ng DNA template, 0.1 µl of 5U Taq Polymerase, and 8.9 µl of sterile distilled water were used and mixed to prepare the Master Mix solution. The quality of DNA was determined by using GelAnalyzer.

The PCR amplification was conducted using TProfessional Standard Thermocycler (Biometra Company). The PCR amplification reaction started with the initial denaturation for 85 seconds at 94°C before proceeding with another 35 cycles of denaturation process which was performed for 35 seconds at 95°C. The process was then followed by annealing which was conducted around 55 seconds at 57°C followed by extension and one cycle of final extension where 90 seconds at 72°C and 10 minutes at 72°C respectively. The PCR products

were analyzed using 1.5% agarose gel electrophoresis and visualized with UV irradiation. The DOC PRINT system (Vilber Lourmat, USA) was used to capture the images.

The purified PCR products were sent for the DNA sequencing to Apical Scientific Sdn. Bhd. (Selangor, Malaysia). Sanger dideoxy sequencing was used to obtain the sequences. The Standard Nucleotide Basic Local Alignment Search Tool (BLAST) network services were used to compare ITS gene sequences with the GenBank sequences database. The National Centre for Biotechnology Information (NCBI) database was used to examine the similarities on the comparison. For depositing ITS sequences, the sequences were prepared in FASTA format. Then, the document was submitted on the GenBank submission portal (<https://submit.ncbi.nlm.nih.gov/subs/genbank/>).

RESULTS AND DISCUSSION

There are 45 isolates of microfungi that were isolated from the infected leaves of cocoa. Each isolate was identified up to the genus level (*Aspergillus* species, *Clonostachys* species, *Colletotrichum* species, *Curvularia* species, *Diaporthe* species, *Fusarium* species, *Lasiodiplodia* species and *Nigrospora* species) based on their morphological characteristics which are macroscopic and microscopic characteristics. All these genera were classified under the phylum of Ascomycota.

Most of the isolates are endophytic microfungi; four genera which are *Diaporthe*, *Colletotrichum*, *Lasiodiplodia* and *Nigrospora* species are commonly known as pathogenic endophytes (Gomes et al., 2013; Wijayawardene et al., 2018; Ma et al., 2018). The isolated microfungi are mostly endophytic fungi where it could penetrate inside of the plants (Compant et al., 2019; Hardoim et al., 2015). A diverse group of microbes known as endophytic fungi are commonly located asymptotically in the plant tissues or the intercellular gaps (Wen et al., 2022).

Macroscopic characteristics such as colony form, elevation, margin, and pigmentation were observed, while the conidia feature including their shape, conidial septa, conidiophore, conidia's length, and width were recorded for microscopic characteristics. In this study, the colony form of all isolates is filamentous since all the isolates are ascomycetes and each genus was varied in pigmentation such as creamy, green, buff, dark green, orange, and black. For conidia features, the shape of conidia was varied such as oval, spherical ellipsoidal, ellipsoid oval, fusiform, cylindrical with rounded ends, and globose. Most of the conidial septa of isolates have no septate except for *Fusarium* species, *Curvularia* species, and *Lasiodiplodia* species that have 2 to 4 septa, 3 septa, and two isolates have 1-septate with striated, respectively.

All isolates have proceeded to the pathogenicity test for 21 days of incubation period. The isolates have shown various responses to the test. There are 12 isolates produced lesions on the cocoa leaves as shown in Table 1 and Figure 1. The genera that produced lesions include *Aspergillus* species, *Diaporthe* species, *Lasiodiplodia* species, and *Nigrospora* species. The lesions varied among different isolates. Some, such as B3417, B3418, and B3437, produced chlorosis with dark, hollow necrotic lesions, while others, including B3419, B3420, B3421, B3423, B3428, and B3444, formed small, dark necrotic spots, as observed on leaves inoculated with these isolates (Figure 1). The remaining isolates exhibited no pathogenicity under the tested conditions.

Table 1. The data of lesion's size of cocoa leaves from 21 days of pathogenicity test.

Isolate	Species identification	Mean lesion size $\pm$ SD (mm)	Pathogenic	Description
B3395	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3396	<i>Fusarium</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3400	<i>Colletotrichum</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3401	<i>Colletotrichum</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3407	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3409	<i>Colletotrichum</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3411	<i>Clonostachys</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3414	<i>Diaporthe stewartii</i>	1.08 $\pm$ 0.36 ^b	Pathogenic	Chlorosis with brown hollow necrotic lesion
B3416	<i>Lasiodiplodia</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3417	<i>Lasiodiplodia theobromae</i>	1.17 $\pm$ 0.29 ^b	Pathogenic	Chlorosis with dark hollow necrotic lesion
B3418	<i>Lasiodiplodia theobromae</i>	3.17 $\pm$ 0.76 ^d	Pathogenic	Chlorosis with dark hollow necrotic lesion
B3419	<i>Lasiodiplodia theobromae</i>	1.67 $\pm$ 0.29 ^b	Pathogenic	Small dark spot necrotic lesion
B3420	<i>Aspergillus fumigatus</i>	1.50 $\pm$ 0.50 ^b	Pathogenic	Small dark spot necrotic lesion
B3421	<i>Diaporthe stewartii</i>	1.67 $\pm$ 0.58 ^b	Pathogenic	Small dark spot necrotic lesion
B3422	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3423	<i>Aspergillus fumigatus</i>	1.67 $\pm$ 0.58 ^b	Pathogenic	Small dark spot necrotic lesion
B3424	<i>Fusarium</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3425	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3426	<i>Aspergillus</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3427	<i>Aspergillus</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3428	<i>Aspergillus aculeatus</i>	1.83 $\pm$ 0.29 ^b	Pathogenic	Small dark spot necrotic lesion
B3429	<i>Aspergillus</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3430	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced

B3431	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3432	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3433	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3434	<i>Clonostachys</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3435	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3436	<i>Colletotrichum</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3437	<i>Nigrospora sphaerica</i>	1.67 ± 0.58 ^b	Pathogenic	Chlorosis with dark hollow necrotic lesion
B3438	<i>Curvularia</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3439	<i>Diaporthe</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3440	<i>Fusarium</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3441	<i>Fusarium</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3442	<i>Clonostachys</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3443	<i>Colletotrichum</i> sp.	0 ^a	Not pathogenic	No lesion produced
B3444	<i>Diaporthe phaseolorum</i>	1.67 ± 0.58 ^b	Pathogenic	Small dark spot necrotic lesion
B3449	<i>Lasiodiplodia theobromae</i>	1.56 ± 0.21 ^b	Pathogenic	Small dark spots with white hollow necrotic
B3456	<i>Colletotrichum</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3457	<i>Colletotrichum</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3464	<i>Fusarium</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3465	<i>Clonostachys</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3467	<i>Fusarium</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3469	<i>Fusarium</i> sp.	0 ^a	Not pathogenic	No lesion observed
B3475	<i>Lasiodiplodia theobromae</i>	2.16 ± 0.36 ^c	Pathogenic	Small dark spot necrotic lesion
Control	-	-	Not pathogenic	No lesion produced

*Different superscript letters in each column indicate that there were significant differences (p<0.05).

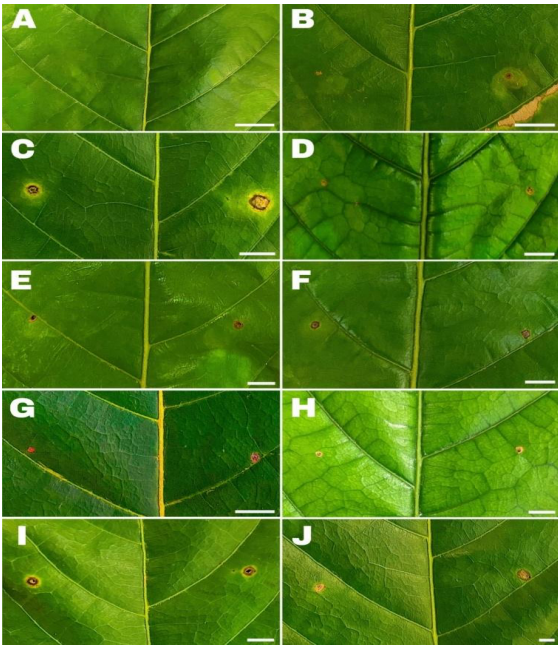


Figure 1. The surface views of cocoa leaves on 21 days after inoculation inoculated with some pathogenic fungal isolates. Control non-wounded (A), isolate B3417 (B), B3418 (C), B3419 (D), B3420 (E), B3421 (F), B3423 (G), B3428 (H), B3437 (I), and B3444 (J). (A) No lesions observed. (B, C, I) Chlorosis with dark hollow necrotic lesions. (D, E, F, G, H, J) Small dark spot necrotic lesion. Scale bar =10 mm.

Based on the lesion sizes produced by the isolates, *Lasiodiplodia theobromae* (B3418) exhibited the highest virulence, with a mean lesion size of 3.17 ± 0.76 mm, while other isolates produced smaller lesions ranging from 1.17 ± 0.29 mm to 2.16 ± 0.36 mm, indicating lower pathogenicity. This study confirms that *L. theobromae* is more virulent than other species associated with cocoa leaf spot disease. Huda-Shakirah et al., 2022) reported, *L. theobromae* was also confirmed to be the causal pathogen of leaf blight, stem canker, and pod rot of *T. cacao* in Malaysia. Furthermore, a study by Ismail et al. (2012) reported that *L. pseudotheobromae* isolates exhibited lower virulence compared to *L. theobromae*, supporting the finding that species within the *Lasiodiplodia* genus vary in their pathogenic potential.

The presence of non-pathogenic isolates suggests that some of these fungi exist as endophytes within cocoa leaves. Many fungal genera identified in this study, including *Diaporthe*

and *Nigrospora*, have been documented as endophytes that can shift to pathogenicity under environmental stress conditions (de Oliveira Costa et al., 2010; Crous et al., 2006). These findings align with previous studies on fungal-host interactions, where endophytic fungi may transition into pathogens when the host plant undergoes physiological stress.

Species identification of the pathogenic isolates was confirmed through ITS sequence analysis. The primary fungal species responsible for leaf spot disease on cocoa included *Aspergillus fumigatus*, *A. aculeatus*, *Diaporthe phaseolorum*, *D. stewartii*, *L. theobromae*, and *Nigrospora sphaerica* (Table 2). Comparing these findings to similar studies provides further evidence of the diversity of fungal pathogens affecting cocoa and underscores the importance of molecular identification in disease management.

Table 2. Accession numbers for the deposited ITS sequences of the pathogenic isolates.

No.	Isolate	Species identification	Accession number of pathogenic isolates
1	B3414	<i>D. stewartii</i>	PP073297
2	B3417	<i>L. theobromae</i>	OQ152095
3	B3418	<i>L. theobromae</i>	OQ152096
4	B3419	<i>L. theobromae</i>	OQ152097
5	B3420	<i>A. fumigatus</i>	OQ152098
6	B3421	<i>D. stewartii</i>	OQ152099
7	B3423	<i>A. fumigatus</i>	OQ152100
8	B3428	<i>A. aculeatus</i>	OQ152101
9	B3437	<i>N. sphaerica</i>	OQ152102
10		<i>D. phaseolorum</i>	OQ152103
	B3444	<i>phaseolorum</i>	OQ152103
11	B3449	<i>L. theobromae</i>	PP073298
12	B3475	<i>L. theobromae</i>	PP073305

CONCLUSIONS

1. This study isolated 45 microfungi species from infected cocoa leaves, identifying them to the genus level, including *Aspergillus*, *Clonostachys*, *Colletotrichum*, *Curvularia*, *Diaporthe*, *Fusarium*, *Lasiodiplodia*, and *Nigrospora*, all classified under the phylum Ascomycota.

2. Pathogenicity tests revealed that 12 isolates produced lesions on cocoa leaves, with *Lasiodiplodia theobromae* (B3418) showing the highest virulence. Other *L. theobromae* isolates exhibited lower virulence, while the remaining isolates were non-pathogenic. These results indicate that some microfungi can transition between endophytic and pathogenic states under stress, contributing to cocoa leaf diseases.
3. The findings provide important insights for developing targeted disease management strategies to improve cocoa productivity. Future research should explore the environmental factors influencing fungal pathogenicity, such as temperature, humidity, and host plant stress responses. Additionally, molecular analyses could help elucidate the genetic mechanisms underlying the transition between endophytic and pathogenic states, aiding in more precise disease control strategies.

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