Comparative analysis of fungal communities in oil palm leaves with leaf spot diseases and fungicide efficacy against key pathogens

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ABSTRACT An unknown leaf spot disease was reported in the nursery of an oil palm estate. A study was conducted to isolate and identify the potential causal agents and to screen the efficacy of different fungicides in suppressing the isolated fungal genera under *in vitro* condition. Disease symptoms were recorded, and healthy and diseased leaflets were collected from the estate's nursery for microbe community analysis. The leaflets were subjected to genomic DNA extraction for Illumina MiSeq sequencing to determine the fungal community in the samples. Additionally, sterilised leaflets were cut and placed on Rose-Bengal chloramphenicol (RBCA) agar for isolating fungal cultures. The DNA of fungal isolates were then extracted, sequenced, and compared with the database using BLAST program. MiSeq sequencing revealed that there were significantly higher relative abundances of *Curvularia* sp. (38.53 %), *Colletotrichum* sp. (14.19 %), and *Thermoascus* sp. (4.79 %) in diseased leaflets compared to healthy leaflets. Different fungicides were screened at the recommended rates using poison food agar assay to test their efficacy in suppressing the potential causal agents. Mancozeb 80% WP (0.188%), propineb 70% WP (0.300%), difenoconazole 23% + propiconazole 23% EC (0.100%), difenoconazole 23% (EC) (0.050%), and prochloraz manganese chloride 50% WP (0.100%) gave 100% mycelial growth inhibitions of *Curvularia oryzae* isolate and two *Colletotrichum* sp. isolates. This study provides insights into the effective fungicides for controlling leaf spot diseases in oil palm nurseries.

KEYWORDS: Oil palm; Leaf spot; *Curvularia*; *Colletotrichum*; MiSeq sequencing; Fungicide
Received 9 December 2024 **Revised** 4 February 2025 **Accepted** 10 February 2025 **In press** 21 March 2025 **Online** 25 March 2025

Original Article

TRANSACTIONS ON SCIENCE AND TECHNOLOGY

INTRODUCTION

Malaysia is the world's second-largest palm oil producer, generating 18.55 million tonnes of CPO in 2023 (Parveez *et al.*, 2024), accounting for 85% of global supply alongside Indonesia. In 2023, palm oil contributed 3.7% to Malaysia's GDP and generated RM 62 billion in export revenue (Parveez *et al.*, 2024). Palm oil remains the largest source of vegetable oils, contributing 39.8% of the global supply, followed by soybean oil and rapeseed oil (Murphy *et al.*, 2021). Its versatility makes it essential not only in food production but also in various non-food applications, such as cosmetics, biofuels, and animal feed (Alimon, 2004; Kushairi *et al.*, 2017; Mekhilef *et al.*, 2011).

Despite its economic importance, oil palm is vulnerable to various pests and diseases. Tropical climates favourable to oil palm also promote phytopathogenic fungi, reducing productivity. (Kittimorakul *et al.*, 2013). At the nursery stage, oil palm leaf spot and leaf blight diseases can cause severe damage, if not death of seedlings (Sunpapao *et al.*, 2018). The disease is initiated by the formation of small, water-soaked lesions, which subsequently become necrotic spots, impairing photosynthetic activity. Over time, individual lesions may coalesce, progressing into extensive leaf blight (Elliott, 2005). In severe cases, affected seedlings may lose a significant number of leaves, rendering them unsuitable for field planting and economically inviable. Several fungal genera including *Curvularia*, *Colletotrichum*, *Pestalotiopsis*, *Cercospora*, *Helminthosporium*, and *Phyllosticta* have been reported to cause leaf spot and leaf blight diseases (Kittimorakul *et al.*, 2013; Mohamed-Azni *et al.*, 2022; Nasehi *et al.*, 2020). In Malaysia, chemical control remains the primary method for managing

leaf spot diseases in oil palm seedlings in the nursery. Alternate spraying of thiram and captan fungicides was recommended in controlling the *Curvularia* leaf blight (Turner & Bull, 1967; MPOB, 2024). In a nursery screening, difenoconazole, copper oxide and propineb reduced the disease intensity of leaf spot diseases caused by *Curvularia* sp. (Susanto & Prasetyo, 2013). Prochloraz and mancozeb fungicides inhibited the growth of *Curvularia* sp. *in vitro* and reduced the leaf spot disease in greenhouse conditions (Kittimorakul *et al*., 2014). Nevertheless, extensive and prolonged use of fungicides may contribute to resistance development.

Next-generation sequencing techniques have proven to be an effective tool in studying microbiomes in natural environments, including those associated with plant diseases (de Assis Costa *et al*., 2018; Azeez *et al*., 2024). Using pyrosequencing analysis, de Assis Costa *et al*. (2018) explored the shifts in fungal communities across different levels of disease severity and identified putative pathogens linked to Fatal Yellowing disease in oil palm leaves. In a recent study, Azeez *et al*. (2024) revealed a high abundance of *Neopestalotiopsis* and *Pseudopestalotiopsis* in healthy pinnae, despite both genera were previously reported to cause oil palm leaf spot diseases (Ismail *et al*., 2017; Mohamed-Azni *et al*., 2022), suggesting an opportunistic pathogenic role. To date, there is a significant gap in knowledge regarding the fungal microbiome associated with leaf spot diseases in oil palm nurseries. Hence, this study aims to (a) characterise the fungal community in oil palm leaves affected by leaf spot diseases using high-throughput Illumina MiSeq sequencing, following the observed outbreak in an oil palm estate, and (b) evaluate the efficacy of commonly used and alternative fungicides in suppressing potential fungal pathogens, given the scanty knowledge concerning their sustained effectiveness after prolonged use.

METHODOLOGY

Field Assessment and Sample Collection

A site visit was conducted in the oil palm nursery at Kekayaan estate, Johor (2° 11' 2.222'' N; 103° 16' 58.415'' E). Symptomatic leaflets displaying leaf spot lesions were randomly collected in triplicates from the infected area, with each sample spaced at least three rows apart. Asymptomatic leaflets were sampled in non-infected area, adjacent to the infected area, using similar protocol. The size of the leaf spots was measured and analysed using Toupview (version 4.11). Samples were brought back to the laboratory for further processing. All the chemicals and reagents were purchased from Thermo Fisher Scientific Inc. (Cleveland, OH, USA) unless specified otherwise.

DNA Extraction and MiSeq Sequencing

Both diseased and healthy leaflets were surface-sterilised by immersing in 70% ethanol for 1 min, followed by 3.125% sodium hypochlorite for 2 mins, 70% ethanol for 1 min, and sterile distilled water (sdH2O) for 3 mins. Sterilised leaflets were ground into powder using liquid nitrogen. Then, the genomic DNA from samples was extracted using HiYieldTM genomic DNA mini kit (plant) (RBC Bioscience Corp., Taipei, Taiwan) according to the manufacturer's instructions. Gel electrophoresis was done to check the DNA bands, and the DNA purity was examined by a SpectraMax ABS Plus spectrophotometer (Molecular Devices LLC., San Jose, CA, USA). The DNA samples were sent to Biozeron Biotechnology Co., Ltd., Shanghai, China for Illumina MiSeq sequencing. The universal fungal barcoding region, internal transcribed spacer (ITS), was selected for the construction of fungal community library for MiSeq sequencing. PCR reactions were performed (initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 5 min) in triplicates of 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each ITS 1 and ITS 2 primer (5

μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, U.S.). Sample libraries were pooled in equimolar and sequenced using paired-end reads (2 × 250/300 bp) on an Illumina MiSeq platform according to the standard protocols.

Processing of Sequencing Data

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.9.1) with the following criteria: (a) The 300 bp reads were truncated at any site with an average quality score of <20 over a 50 bp sliding window, and any truncated reads shorter than 50bp were discarded; (b) Exact barcode matching was used, with up to 2 nucleotide mismatches allowed in primer matching. Reads containing ambiguous characters were removed. (c) Only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded.

Pure culture Isolation

Sterilised leaflets were excised into 1cm × 1cm segments and subsequently placed onto Rose-Bengal chloramphenicol agar (RBCA). Infected leaflets were cut at the margins of the leaf spot to facilitate the growth of potential fungal causal agents. Inoculated plates were incubated at 24°C in the dark for 7 days and any emergence of fungal colonies was recorded. Distinctive fungal colonies were subcultured onto a new RBCA plate using a sterile inoculation needle to obtain a pure culture. The pure cultures were subsequently maintained on malt extract agar (MEA).

Molecular Identification of Fungal Isolate

The genomic DNA from pure culture was extracted and was further analysed by PCR to amplify the ITS 1 and ITS 4 regions using the primer pair '5'- TCCGTAGGTGAACCTGCG-3' and 5'-TCCTCCGCTTATTGATATGC-3'. The PCR reaction was performed (initial denaturation of 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min) in a 25 μl reaction mixture containing 1 μl from each primer (10 uM), 12.5 μl Cybergreen DNA Polymerase Master Mix (Nuclix Biosolutions Sdn. Bhd., Selangor, Malaysia), 2 μl of DNA template and 8.5 μl of nuclease-free water. The amplicons were subjected to electrophoresis on 1.5% gel at (80V) for one hour and were visualised using a Slite 600 gel imaging system (Pacific Image Electronics Co., Ltd., Taiwan). Subsequent purification and sequencing of the amplicons were conducted by Nuclix Biosolutions Sdn. Bhd. (Selangor, Malaysia). The nucleotide sequences were analysed using MEGA (version 11) and compared with GenBank database using Basic Local Alignment Search Tool (BLAST) program for identification.

Poison Food Bioassay

Twelve different fungicides with either multi-site or single-site mode of action (MoA) were tested at two different rates: (a) recommended rates based on the pesticide labels, and (b) additional rates to compare across pesticides at equal rates (Table 1) to determine their efficacy in suppressing the potential leaf spot pathogens *in vitro*. Poison food media was prepared as follows: (a) MEA was prepared based on the product recommendation and was autoclaved at 121°C for 15 mins. (b) After autoclaving, fungicides were added into MEA at their respective rates (Table 1) along with 0.01% chloramphenicol and left solidified. The control treatment was prepared using MEA with 0.01% chloramphenicol only. Each distinctive fungus genus was freshly prepared by subculturing onto MEA. A 5 mm mycelial plug of fungal isolate was transferred to the centre of poison food media and incubated at 24°C in the dark for 7 to 14 days. The mycelial growth of fungal isolates was measured

in four different directions from the mycelial plug and recorded. The percent inhibition of radial growth (PIRG) (%) was calculated based on the formula:

$$PIRG\ (\%) = \frac{M_c - M_p}{M_c} \times 100\%$$

where M_c Mycelial growth in control treatment and M_p Mycelial growth in poison food media.

Table 1. List of fungicidal active ingredients (A.I.) with multisite or single site modes of action (MoA) tested at two different rates (recommended rate and additional rate for comparison) for suppressing the potential causal agents *in vitro*.

Fungicide A.I.	Rate 1	Rate 2
Multisite MoA		
Mancozeb 80% WP	0.125 **	0.188 *
Propineb 70% WP	0.300 *	0.188 **
Chlorothalonil 50% WP	0.125 **	0.188 *
Thiram 80% WP	0.125 **	0.188 *
Zineb 80% WP	0.125 **	0.188 *
Captan 80% WG	0.125 *	0.188 **
Single site MoA		
Picoxystrobin 17.9% + Cyproconazole 7.17% SC	0.100 **	0.180 *
Difenoconazole 23% + Propiconazole 23% EC	0.100 *	0.180 **
Difenoconazole 23.23% EC	0.050 *	0.180 **
Prochloraz manganese chloride 50% WP	0.100 *	0.180 **
Carbendazim 42.0% SC	0.100 *	0.180 **
Azoxystrobin 22.94% SC	0.050 *	0.180 **

* refers to recommended rate, while ** refers to additional rate for comparison.

Bioinformatics and Statistical Analysis

Operational Taxonomic Units (OTUs) were clustered using 97% similarity as the lower limit using Usearch (version 10 http://drive5.com/uparse/). The taxonomy of each ITS gene sequence was analysed by Ribosomal Database Project Classifier (Version 2.2 http://sourceforge.net/projects/rdp-classifier/) against the Unite (Release 8.2 http://unite.ut.ee/index.php) ITS database using confidence threshold of 0.97. For α-diversity analysis, rarefaction curve using Shannon-Wiener index was calculated using custom R scripts to assess species richness. Community richness was assessed using Chao estimator and Ace, while community diversity was characterised using Shannon index and Simpson index. Principal coordinate analysis (PCoA) with weighted UniFrac distances was calculated using the custom R scripts. The difference in the microbial community in the samples was compared using analysis of similarity (ANOSIM), permutational multivariate analysis (Adonis), and Linear Discriminate Analysis Effect Size (LEfSe). Independent sample t-test was used to compare the α-diversity, while Wilcoxon rank-sum test was used to differentiate the relative abundance of fungal taxa between healthy and diseased leaflets.

RESULT AND DISCUSSION

Two different lesions or leaf spots were observed from the symptomatic leaflets (Figure 1): (a) black, circular to irregular-shaped lesions with or without orange to yellow halos; diameter: 0.07 – 0.59 cm, and (b) brown, elliptical-shaped lesions with bright-yellow halos; diameter: 0.08 – 0.77 cm. These symptoms were not present on the asymptomatic leaflets.

Figure 1. Leaf spot disease symptoms on oil palm seedlings. (A, B) Diseased leaflets; (C) Healthy leaflets. Red arrows indicate black, circular to irregular-shaped lesions with or without orange to yellow halos (diameter: 0.07 – 0.59 cm), while blue arrows indicate brown, elliptical-shaped lesions with bright-yellow halos (diameter: 0.08 – 0.77 cm). Scale bar: 1 cm.

The total number of ITS reads obtained from six samples, after filtering mismatch sequences, was 373,494 reads. These reads were further clustered into 529 OTUs at a minimum of 97% similarity. The Shannon-Wiener curve exhibited a plateau to the right (data not shown), suggesting that the sampling effort captured a substantial portion of the fungal diversity and further sampling is unlikely to yield new species. Both Chao (p = 0.021) and Ace (p = 0.023) indices were significantly higher in healthy leaflets than in diseased leaflets, suggesting a greater abundance of fungal species in healthy leaflets (Table 2). Additionally, Shannon index (p = 0.015) and Simpson index (p = 0.011) were also significantly higher in healthy leaflets, indicating a higher taxonomic diversity of fungus compared to diseased leaflets. These results imply that the fungal community in healthy leaflets is more diverse, while diseased leaflets are more likely to be dominated by specific fungal species.

Table 2. The α-diversity of the fungal ITS sequencing libraries from MiSeq sequencing analysis. The community richness was assessed using Chao estimator and Ace, while the community diversity was characterised using Shannon index and Simpson index.

Sample type	Number of OTUs	Chao	Ace	Shannon	Simpson
Diseased leaflets	263.33 ± 10.48	292.53 ± 13.58	289.17 ± 13.28	2.239 ± 0.087	0.769 ± 0.029
Healthy leaflets	329.00 ± 11.37	365.09 ± 5.47	368.87 ± 2.54	3.962 ± 0.265	0.950 ± 0.016
p-value	0.013	0.021	0.023	0.015	0.011

*Data were presented as mean ± standard deviation. Mean values in the column differ significantly if p-value is smaller than 0.05 based on independent samples t-test.

PCoA was conducted using weighted UniFrac distance to analyse the dissimilarities in the fungal communities between healthy leaflets (KH1 - 3) and diseased leaflets (KD1 - 3) (Figure 2). The fungal

communities of KD1 and KD2 were more closely related, while KD3 was slightly different from both KD1 and KD2. The fungal communities in healthy leaflets (KH1 – KH3) were not closely related to those in diseased leaflets but were also distinct from each other in both taxa composition and relative abundance. In PCoA with weighted UniFrac distance, PC1 alone explains 68% of the variances, followed by PC2 (13%). Both ANOSIM (p = 0.1) and Adonis (p = 0.1) indicated no significant differences in the fungal communities between healthy and diseased leaflets. Both diseased and healthy groups may possess comparable taxa assemblages, which could be due to the sampling of both groups at close geographical proximity, potentially accounting for the lack of a significant difference between them. Furthermore, small sample size may also contribute to the lack of statistical significance observed in the analysis.

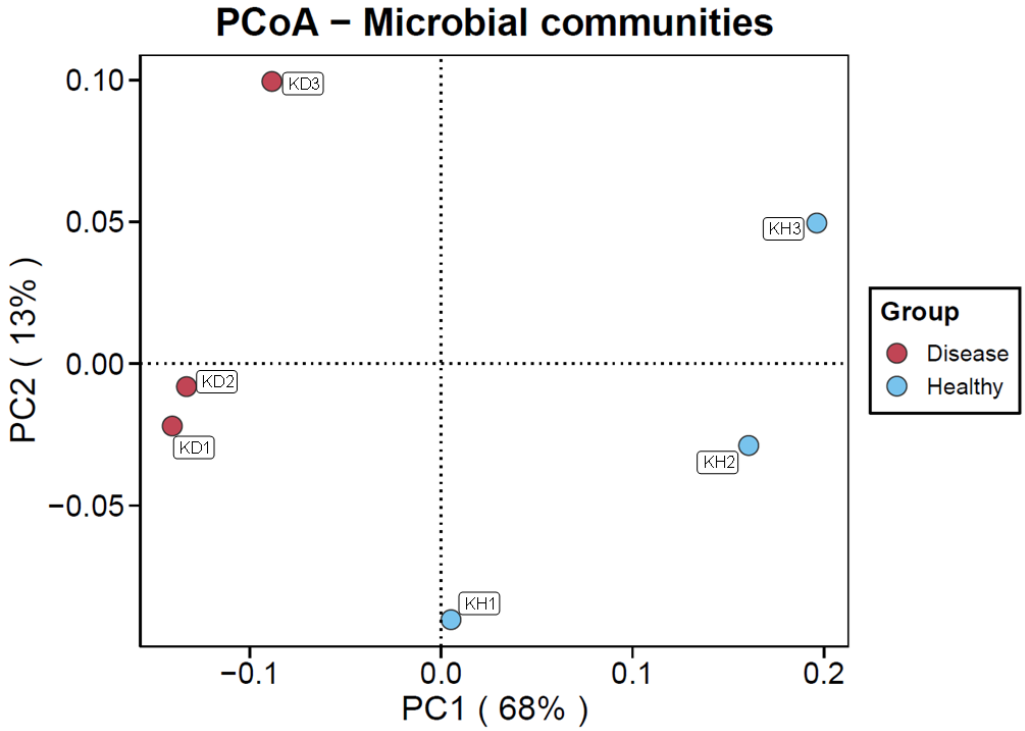

Figure 2. Principal Coordinate Analysis (PCoA) of fungal communities based on weighted UniFrac distance. Points representing healthy leaflets (KH1–3) and diseased leaflets (KD1–3) are shown in blue and red, respectively, to highlight their clustering patterns. PC1 explains 68% of the variance, followed by PC2 (13%). Statistical analysis (ANOSIM, p-value = 0.1; Adonis, p-value = 0.1) revealed no significant differences between healthy and diseased leaflets.

The OTUs were further classified into different taxa and their relative abundances were estimated between healthy and diseased leaflets. The 10 most dominant fungal genera were analysed by MiSeq sequencing as shown in Figure 3. In the diseased leaflets, significantly higher relative abundance of *Curvularia* sp. was recorded (38.53 %) compared to healthy leaflets (3.01 %) (p = 0.001). Besides, the relative abundances of *Colletotrichum* sp. (diseased: 14.19 %, healthy: 2.21 %, p = 0.001) and *Thermoascus* sp. (diseased: 4.79 %, healthy: 1.04 %, p = 0.001) were also significantly higher in diseased leaflets compared to healthy leaflets. Higher relative abundances of *Paraphaeosphaeria* sp. (diseased: 13.55 %, healthy: 5.88 %, p = 0.401), *Pyricularia* sp. (diseased: 8.06 %, healthy: 0.98 %, p = 0.201, and *Microsphaeropsis* sp. (diseased: 4.87 %, healthy: 0.67 %, p = 0.601) were obtained in diseased leaflets but were not statistically significant compared to healthy leaflets. The results were congruent with LEfSe (Figure 4), where significantly higher (LDA score > 2) relative abundances of *Curvularia* sp., *Colleotrichum* sp., and *Thermoascus* sp. were obtained in diseased leaflets compared to healthy leaflets.

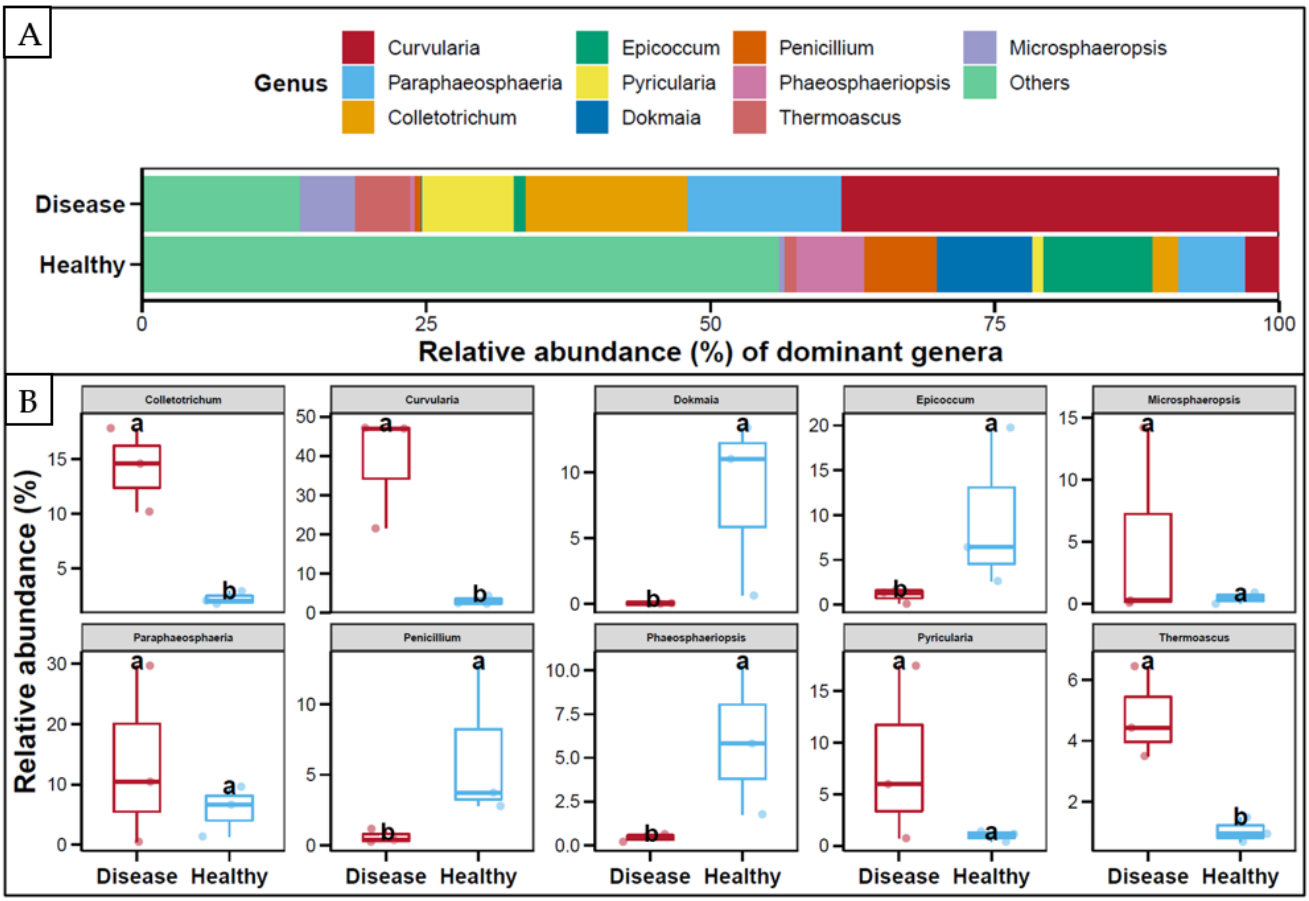

Figure 3. Relative abundances (%) of 10 most dominant fungal genera between healthy and diseased leaflets in (A) bar plot; and (B) box plot. Box plots with the same letter indicate no significant difference in relative abundance between healthy and diseased leaflets, based on the Wilcoxon rank-sum test at *p*-value = 0.05.

Both *Curvularia* and *Colletotrichum* genera have been widely reported to cause leaf spot and leaf blight diseases (Anuar & Ali, 2022; Kittimorakul *et al*., 2013; Priwiratama *et al*., 2024a). A study of leaf spot diseases across Indonesia revealed that *Curvularia* and *Pestalotiopsis* were dominant, with their prevalence varying by region (Priwiratama *et al*., 2024b). Accurate identification of fungal pathogens is essential for effective decision-making and the implementation of appropriate control measures. In this current study, *Curvularia* remains the predominant genera potentially responsible for the leaf spot diseases, followed by *Colletotrichum*. However, these findings are based on the oil palm nursery in Kekayaan estate, Johor, and the pathogens causing leaf spot diseases in other regions of Malaysia could possibly be different.

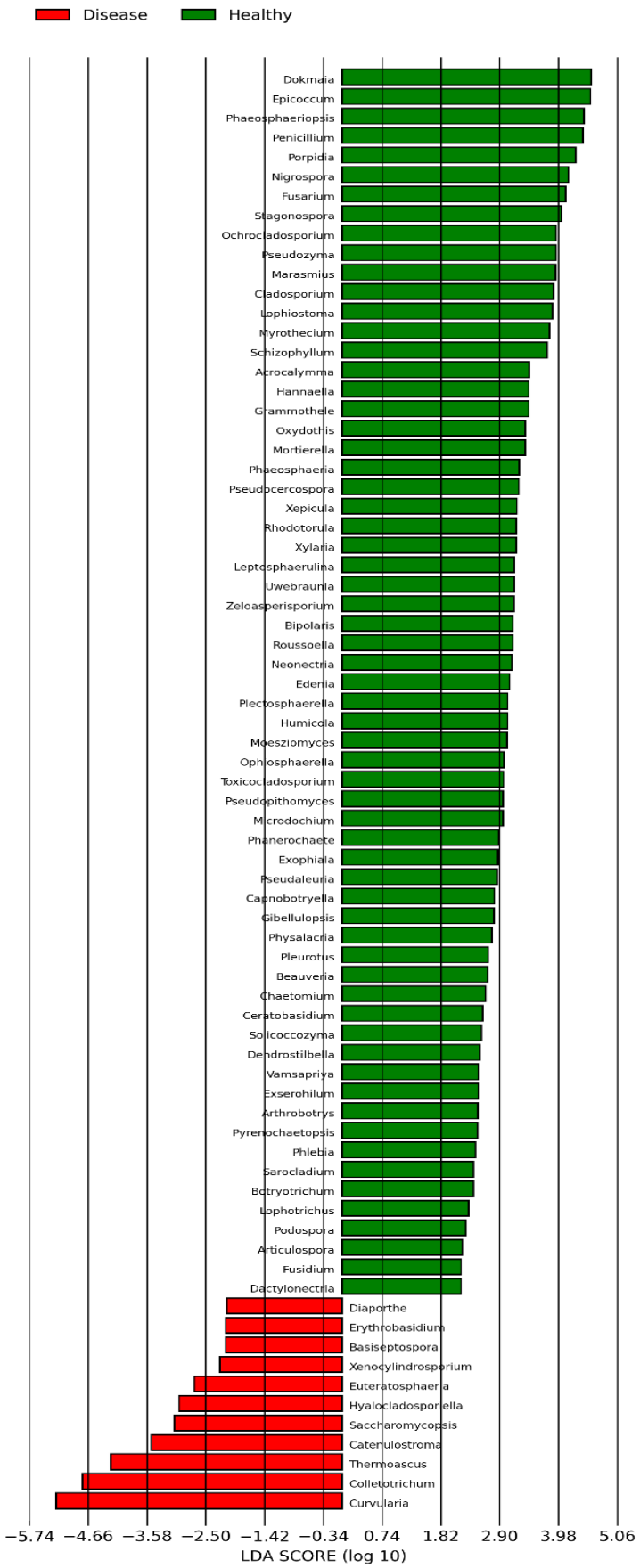

Figure 4. Linear Discriminate Analysis Effect Size (LEfSe) plot showing difference in fungal taxa between healthy and diseased leaflets at genus level. Each bar represents a genus with significant differences in abundance between groups. Positive LDA (linear discriminate analysis) scores indicate taxa enriched in the healthy leaflets and vice versa.

Based on the previously mentioned analyses of species abundance (Chao and Ace indices) and diversity (Shannon and Simpson indices), the results demonstrated a significantly higher fungal population and a more diverse mycobiome in the healthy leaflets compared to diseased leaflets. In this study, several fungal genera, such as *Epicoccum*, *Chaetomium*, and *Cladosporium* were revealed to be significantly higher in healthy leaflets compared to diseased leaflets according to LEfSe. During the site visit, healthy leaflets were selected based on the absence of visible leaf spot or leaf blight symptoms. Consequently, the fungal genera identified in healthy leaflets, which demonstrate significant differences in relative abundance compared to diseased leaflets, possess endophytic capability within the plant host and may exhibit antagonistic ability against leaf spot pathogens. Certain endophytes have the potential to secrete antimicrobial metabolites and proteins or induce structural changes in plant cells that enhance resistance to pathogen penetration (Latz *et al*., 2018). *Epicoccum nigrum*, a facultative endophyte, enhanced the root dry weight of sugarcane and produced bioactive compounds that inhibited the mycelial growth of various plant pathogens (Fávaro *et al*., 2012). *Chaetomium* sp. was revealed to be a promising biocontrol agent, with various studies reported to protect crops against fungal pathogens including *Phytophthora infestans*, *Bipolaris sorokiniana* and *Setosphaeria turcica* (Shanthiyaa *et al*., 2013; Zhang *et al*., 2013; Yue *et al*., 2018). *Cladosporium*, a cosmopolitan species, is widely recognized for its effectiveness against various arthropod pests and phytopathogenic fungi (Islam, 2022). Although the functional ecology of these taxa in oil palm leaves is not well understood, a diverse fungal population may indicate a protective role to its host, promoting plant health and durability against potential leaf spot pathogens. These fungal genera could also be explored as biocontrol agents and integrated into control measures for sustainable disease management of leaf spot diseases.

A total of 17 fungal colonies were isolated from diseased leaflets. Each isolate was identified using molecular techniques, where the ITS region was amplified and sequenced. Based on the sequencing result, five *Colletotrichum* sp. isolates and four *Curvularia oryzae* isolates were successfully identified. This suggests that both *C. oryzae* and *Colletotrichum* sp. were present in the diseased leaflets, consistent with the MiSeq sequencing results. Additionally, one *Microsphaeropsis arundinis* isolate, one *Paraphaeosphaeria arecacearum* isolate, two *Xylaria feejeensis* isolates, two *Diaporthe* sp. isolates, one *Stilbohypoxylon elaeidis* isolate, and one *Rosellinia necatrix* isolate were also isolated and identified (Table 3). The fungal isolates *Microsphaeropsis arundinis* and *Xylaria feejeensis* were previously reported to be pathogenic to oil palm leaves (Mohamed-Azni *et al*., 2022).

Table 3. The identified fungal genera from symptomatic leaflets based on nucleotide sequences comparison with GenBank database.

Fungal genera	No. of isolates
Colletotrichum sp.	5
Curvularia oryzae	4
Paraphaeosphaeria arecacearum	1
Microsphaeropsis arundinis	1
Xylaria feejeensis	2
Diaporthe sp.	2
Stilbohypoxylon elaeidis	1
Rosellinia necatrix	1
Total	**17**

In fungi, the nuclear ribosomal RNA (rRNA) internal transcribed spacer (ITS) region serves as a universal DNA barcoding marker for the vast majority of fungal species (Schoch *et al*., 2012). The entire ITS region is approximately 600 bp in length and includes two variable spacers, ITS-1 and ITS-

2. These variable spacers are flanked by highly conserved rRNA genes (18S, 5.8S, and 28S rRNA), allowing the design of universal primers to amplify the ITS-1, ITS-2, or the entire ITS region (Xu, 2016). However, it is ineffective for distinguishing closely related species with minimal sequence variation, such as *Colletotrichum*. Several molecular markers, including the mating-type locus MAT1-2-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-tubulin (TUB2), have been used to differentiate among *Colletotrichum* species (Anderson dos Santos Vieira *et al*., 2019). In this study, all five *Colletotrichum* sp. were successfully identified until genus level using ITS primers; further efforts would be needed to incorporate other molecular markers for species-level resolution.

Thermoascus sp. was not isolated despite its significantly high relative abundance in diseased leaflets. *Thermoascus* sp. belongs to a group of thermophilic fungi, which are commonly found in self-heating environments associated with decaying plant materials (de Oliveira & Rodrigues, 2019; Witfeld *et al*., 2021). It is hypothesised that the temperature increase from the exothermic metabolism of fungal pathogens provides a conducive environment for proliferation of *Thermoascus* sp., suggesting its role as a saprophyte. Isolation of thermophilic fungi remains challenging as they may be overgrown by fast-growing species (Witfeld *et al*., 2021). In this study, isolation of fungal species was conducted at 24°C, which may not be conducive for certain *Thermoascus* sp. that require a minimum growth temperature of 30°C (Deploey, 1999). Hence, specific isolation techniques will be required to isolate *Thermoascus* sp. and evaluate their pathogenicity in oil palm leaves.

Fungal isolates from different genera were selected to assess their sensitivity to various fungicides. Nine isolates, including *Curvularia oryzae* K2, *Microsphaeropsis arundinis* K3, *Colletotrichum* sp. K5, *Colletotrichum* sp. K23, *Paraphaeosphaeria arecacearum* K7, *Diaporthe* sp. K9, *Rosellinia necatrix* K12, *Xylaria feejeensis* K19, and *Stilbohypoxylon elaeidis* K27, were inoculated onto poison food agar containing different fungicides to evaluate their growth rate (Table 4). Among fungicides with multi-site modes of action (MoA), a 100% mycelial growth inhibition of *C. oryzae* K2 was recorded using mancozeb 80% WP (0.188%). and propineb 70% WP (0.300%) (Table 4). However, thiram (80% WP) only suppressed the growth of *C. oryzae* K2 by 83.5% and *Colletotrichum* sp. K23 by 96.2% *in vitro* at the recommended rate (0.188%) Despite having multi-site modes of action, zineb 80% WP (0.188%), captan 80% WG (0.125%) and chlorothalonil 70% WP (0.188%) only suppressed the growth of *C. oryzae* K2 by 88.5%, 66.0%, and 19.4%, respectively, at recommended rates.

Among single-site MoA fungicides, three, particularly difenoconazole 23% + propiconazole 23% EC, difenoconazole 23.23% EC, and prochloraz manganese chloride 50% WP completely suppressed the growth of all fungal isolates tested at their recommended rates (0.100%, 0.050%, and 0.100%, respectively). Picoxystrobin 17.9% + cyproconazole 7.17% SC fully suppressed the mycelial growth of *C. oryzae K2* but partially inhibited the growth of *Colletotrichum* sp. K5 and K23. At recommended rates, both azoxystrobin 22.94% SC (0.050%) and carbendazim 42.0% SC (0.100%) only suppressed the growth of *C. oryzae* by 68.5% and 30.2%, respectively.

Fungicides with multi-site activity are generally recommended, as they have a low risk of developing resistance. Although thiram and captan fungicides were recommended for controlling leaf blight caused by *Curvularia* sp. (Turner & Bull, 1967; MPOB, 2024), current results showed that *C. oryzae* K2 is less sensitive to both fungicides. Repeated and prolonged application of the same pesticide can potentially induce resistance in the pathogen, thereby diminishing its effectiveness. Alternatively, propineb 70% WP and mancozeb 80% WP at 0.188% and 0.300%, respectively, could be used in suppressing the potential fungal causal agents, particularly leaf spot diseases caused by *C. oryzae* and *Colletotrichum* sp. Both fungicides were also effective in controlling other fungal genera isolated from the diseased leaflets.

Table 4. Percentage of radial growth inhibition (PIRG) (%) of fungal isolates subjected to twelve different fungicides *in vitro*.

Fungicide	Rate (%)	Percentage of radial growth inhibition (PIRG) (%) ¥								
		K2	K5	K23	K3	K7	K9	K12	K19	K27
Multi-site Modes of Action										
Mancozeb 80% WP	0.125 **	94	100	100	100	100	100	100	100	100
	0.188 *	100	100	100	100	100	100	100	100	100
Propineb 70% WP	0.188 **	96.8	100	85.2	100	100	100	100	100	100
	0.300 *	100	100	100	100	100	100	100	100	100
Chlorothalonil 50% WP	0.125 **	16.1	62.0	45.7	71.7	79.3	74.8	80.4	42.2	100
	0.188 *	19.4	71.5	55.1	75.9	84.9	89.4	84.4	74.5	100
Thiram 80% WP	0.125 **	88.3	100	89.5	90.1	100	96.3	100	100	100
	0.188 *	83.5	100	96.2	100	100	97.5	100	100	100
Zineb 80% WP	0.125 **	76.7	100	50.6	100	92.0	64.6	100	86.7	100
	0.188 *	88.5	100	52.6	100	100	59.9	100	100	100
Captan 80% WG	0.125 *	66.0	94.9	73.8	89.3	89.7	68.5	66.4	68.9	76.7
	0.188 **	71.2	96.3	83.1	93.7	93.0	63.0	67.9	67.9	95.6
Single site Mode of Action										
Difenoconazole 23.23% EC	0.050 *	100	100	100	100	100	100	100	100	100
	0.180 **	100	100	100	100	100	100	100	100	100
Prochloraz manganese chloride 50% WP	0.100 *	100	100	100	100	100	100	100	100	100
	0.180 **	100	100	100	100	100	100	100	100	100
Carbendazim 42.0% SC	0.100 *	30.2	100	94.2	100	100	100	100	100	37.3
	0.180 **	44.8	100	93.8	100	100	100	100	100	42.6
Azoxystrobin 22.94% SC	0.050 *	68.5	79.0	54.6	87.5	86.3	34.9	100	50.3	100
	0.180 **	53.5	83.7	60.0	100	90.4	64.6	100	49.7	100
Picoxystrobin 17.9% + Cyproconazole 7.17% SC	0.100 **	100	85.8	70.3	100	100	100	100	100	100
	0.180 *	100	94.4	91.8	100	100	100	100	100	100
Difenoconazole 23% + Propiconazole 23% EC	0.100 *	100	100	100	100	100	100	100	100	100
	0.180 **	100	100	100	100	100	100	100	100	100

* Refers to recommended rate, while ** refers to additional rate for comparison.

¥ Fungal isolates tested were - K2: *Curvularia oryzae*; K5 and K23: *Colletotrichum* sp.; K3: *Microsphaeropsis arundinis*; K7: *Paraphaeosphaeria arecacearum*; K9: *Diaporthe* sp.; K12: *Rosellinia necatrix*; K27: *Stilbohypoxylon elaeidis*; and K19: *Xylaria feejeensis*.

Besides, fungicides with single-site MoA were also selected for their efficacy on isolated fungal genera. It was found that demethylation inhibitor (DMI) fungicides (FRAC group 3), particularly difenoconazole 23% + propiconazole 23% EC, difenoconazole 23.23% EC, and prochloraz manganese chloride 50% WP were effective against *C. oryzae* and *Colletotrichum* sp. as well as other isolated fungal genera. On the contrary, quinone outside inhibitor (QoI) fungicide using azoxystrobin (FRAC group 11) and benzimidazole fungicide using carbendazim (FRAC group 1) were less effective in suppressing *C. oryzae*. The results were congruent with Gholve *et al*. (2018), in which difenoconazole and propiconazole were the most effective in inhibiting *Curvularia lunata*, while both azoxystrobin and carbendazim were less effective. *Colletotrichum godetiae* also exhibited higher sensitivity to difenoconazole, propiconazole, and prochloraz-manganese, while demonstrating lower sensitivity to azoxystrobin (Peng *et al*., 2022). Yet, despite carbendazim was ineffective in suppressing *C. oryzae*, it showed high inhibition when tested against *Colletotrichum* K5 (100%) and K23 (94.2%). Ramani *et al*. (2015) discovered that carbendazim was effective against *Colletotrichum gloeosporioides*, a causal agent of banana anthracnose. Highest inhibition zone of *Colletotrichum capsica* was also observed *in vitro* using carbendazim, compared to captan (multi-site MoA) (Nongmaithem & Rebika, 2019). Future work will involve screening all the effective fungicides to evaluate their efficacy *in vivo*.

Despite having high efficacy, fungicides from FRAC group 3, (difenoconazole 23.23% EC and prochloraz manganese chloride 50% WP) are associated with a moderate risk of resistance development (FRAC, 2024). Alternating fungicides with different modes of action and integrating them with other control measures may be necessary to delay resistance development. In addition to chemical treatments, biological control agents such as *Streptomyces*, *Bacillus* and *Trichoderma*, and plant extracts have also emerged as a promising approach for suppressing *Curvularia* leaf spot in a nursery setting (Sunpapao *et al*., 2018; Febriani & Kasiamdari, 2023; Hernández-Navarro *et al*., 2023). Besides, cultural practices, such as the regular culling or removal of infected seedlings, can effectively mitigate the spread of pathogen inoculum. Pruning of senescent, diseased foliage may contribute to disease suppression, but excessive pruning could negatively impact plant growth and delay flowering (Corley & Tinker, 2015). An integrated disease management approach, combining chemical, biological, and cultural practices, is crucial for achieving long-term, effective control of leaf spot diseases, while also mitigating the risk of pathogen resistance to synthetic fungicides.

CONCLUSION

Illumina MiSeq sequencing revealed higher relative abundances of *Curvularia* and *Colletotrichum* in oil palm leaflets with leaf spot disease compared to healthy leaflets. Seventeen fungal isolates were isolated from diseased leaflets, including *Curvularia oryzae*, *Colletotrichum* sp., *Microsphaeropsis arundinis*, *Xylaria feejeensis*, with *Curvularia* and *Colletotrichum* being the most prevalent genera. Fungicide screening demonstrated that 100% mycelial growth inhibition of *C. oryzae* and two *Colletotrichum* isolates were observed using mancozeb 80% WP (0.188%), propineb 70% WP (0.300%), difenoconazole 23% + propiconazole 23% EC (0.100%), difenoconazole 23% (EC) (0.050%), and prochloraz manganese chloride 50% WP (0.100%).

ACKNOWLEDGEMENTS

The author would like to thank our Principals, Boustead Plantation Berhad and Kuala Lumpur Kepong Berhad for their permission to publish these data and for their financial backing given to the research activities leading to this article.

REFERENCES

[1] Alimon, A. R. 2004. The nutritive value of palm kernel cake for animal feed. *Palm Oil Developments*, 40(1), 12-14.

[2] Anuar, M. A. S. S. & Ali, N. S. 2022. Significant oil palm diseases impeding global industry: A review. *Sains Malaysiana,* 51(3), 707-721.

[3] Anderson dos Santos Vieira, W., Alves Bezerra, P., Carlos da Silva, A., Silva Veloso, J., Paz Saraiva Câmara, M. & Patrick Doyle, V. 2019. Optimal markers for the identification of *Colletotrichum* species. *Molecular Phylogenetics and Evolution,* 143, 106694.

[4] Azeez, A. A., Esiegbuya, D. O., Lateef, A. A. & Asiegbu, F. O. 2024. Mycobiome analysis of leaf, root, and soil of symptomatic oil palm trees (*Elaeis guineensis* Jacq.) affected by leaf spot disease. *Frontiers in Microbiology,* 15, 1422360.

[5] Corley, R. H. V. & Tinker, P. B. 2015. *The Oil Palm* (5th edition). Oxford: Blackwell Science.

[6] de Assis Costa, O. Y., Tupinambá, D. D., Bergmann, J. C., Barreto, C. C. & Quirino, B. F. 2018. Fungal diversity in oil palm leaves showing symptoms of Fatal Yellowing disease. *PloS ONE,* 13(1), e0191884.

[7] de Oliveira, T. B. & Rodrigues, A. 2019. Ecology of Thermophilic Fungi. *In*: Tiquia-Arashiro, S. M. & Grube, M. (Eds.). *Fungi in Extreme Environments: Ecological Role and Biotechnological Significance*, Cham: Springer. pp 39-57.

[8] Deploey, J. J. 1999. Temperature and nutritional requirements of *Thermoascus aurantiacus*. *Journal of the Pennsylvania Academy of Science,* 72(2), 68-72.

[9] Elliott, M. L. 2005. *Leaf spots and leaf blights of palm* (https://citeseerx.ist.psu.edu/document?repid=rep1&type=pdf&doi=f84534ccc0e46b4f0c61c818fb62d7b904fede14). Last accessed on 1 November 2024.

[10] Fávaro, L. C. d. L., Sebastianes, F. L. d. S. & Araújo, W. L. 2012. *Epicoccum nigrum* P16, a sugarcane endophyte, produces antifungal compounds and induces root growth. *PloS ONE,* 7(6), e36826.

[11] Febriani, A. V. & Kasiamdari, R. S. 2023. Identification of *Curvularia eragrostidis* (Henn.) JA Mey. the leaf spot pathogen of oil palm (*Elaeis guineensis* Jacq.) and it's control by false elder (*Peronema canescens* Jack) leaf extract. *International Journal of Oil Palm*, 6(2), 25–34.

[12] FRAC (Fungicide Resistance Action Committee). 2024. *FRAC Code List©* 2024: Fungal Control Agents Sorted By Cross-resistance Pattern and Mode of Action (Including Coding for FRAC Groups on Product Labels)* (https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2024.pdf). Last accessed on 14 November 2024.

[13] Gholve, V. M., Sawade, B. R., Kalpande, H. V. & Das, I. K. 2018. Efficacy of new fungicides and bioagents against grain mold fungi. *Journal of Mycopathological Research,* 56(1), 41-49.

[14] Hernández-Navarro, E., Agustín-Maravilla, G. Á., Sánchez-Rangel, J. C., Valadez-Ramírez, P. & Chan-Cupul, W. 2023. *In vitro* evaluation of biological fungicides against *Curvularia eragrostidis*, a phytopathogenic fungus of pineapple crop. *Mexican Journal of Phytopathology,* 41(1), 93-111.

[15] Islam, M. T. 2022. Current status and future prospects of *Cladosporium* sp., a biocontrol agent for sustainable plant protection. *Biocontrol Science,* 27(4), 185-191.

[16] Ismail, S. I., Zulperi, D., Norddin, S. & Ahmad-Hamdani, S. 2017. First report of *Neopestalotiopsis saprophytica* causing leaf spot of oil palm (*Elaeis guineensis*) in Malaysia. *Plant Disease,* 101(10), 1821-1822.

[17] Kittimorakul, J., Pornsuriya, C., Sunpapao, A. & Petcharat, V. 2013. Survey and incidence of leaf blight and leaf spot diseases of oil palm seedlings in Southern Thailand. *Plant Pathology Journal,* 12(3), 149-153.

[18] Kittimorakul, J., Petcharat, V. & Chuenchitt, S. 2014. Chemical and biological control of *Curvularia oryzae* leaf spot of oil palm. *Songklanakarin Journal of Plant Science,* 1(1), 39-47.

[19] Kushairi, A., Singh, R. & Ong-Abdullah, M. 2017. The oil palm industry in Malaysia: Thriving with transformative technologies. *Journal of Oil Palm Research,* 29(4), 431-439.

[20] Latz, M. A. C., Jensen, B., Collinge, D. B. & Jørgensen, H. J. L. 2018. Endophytic fungi as biocontrol agents: Elucidating mechanisms in disease suppression. *Plant Ecology & Diversity,* 11(5-6), 555-567.

[21] Mekhilef, S., Siga, S. & Saidur, R. 2011. A review on palm oil biodiesel as a source of renewable fuel. *Renewable and Sustainable Energy Reviews,* 15(4), 1937-1949.

[22] Mohamed-Azni, I. N. A., Sritharan, K., Ho, S.-H., Roslan, N. D., Arulandoo, X. & Sundram, S. 2022. Isolation, identification and pathogenicity of fungi associated with leaf blotches in *Tenera* x *Tenera* (TxT) variety of oil palm in Malaysia. *Journal of Plant Pathology,* 104, 167-177.

[23] MPOB (Malaysian Palm Oil Board). 2024. *Nursery Leaf Spot Diseases* (http://sawitsecure.mpob.gov.my/nursery-leaf-spot-disease/). Last accessed on 21 November 2024.

[24] Murphy, D. J., Goggin, K. & Paterson, R. R. M. 2021. Oil palm in the 2020s and beyond: Challenges and solutions. *CABI Agriculture and Bioscience*, 2(39), 1-22.

[25] Nasehi, A., Sathyapriya, H. & Wong, M. 2020. First report of leaf spot on oil palm caused by *Phyllosticta capitalensis* in Malaysia. *Plant Disease*, 104(1), 288.

[26] Nongmaithem, N. & Rebika, T. 2019. Screening of fungicides against leaf spot of turmeric caused by *Colletotrichum capsici*. *The Pharma Innovation Journal,* 8(12), 12-14.

[27] Parveez, G. K. A., Leow, S.-S., Kamil, N. N., Madihah, A. Z., Ithnin, M., Ng, M. H., Yusof, Y. A. & Idris, Z. 2024. Oil palm economic performance in Malaysia and R&D progress in 2023. *Journal of Oil Palm Research,* 36(2), 171-186.

[28] Peng, K., Pan, Y., Tan, T., Zeng, X., Lin, M., Jiang, S., Zhao, Z., Tian, F. & Zhao, X. 2022. Characterization and fungicide sensitivity of *Colletotrichum godetiae* causing sweet cherry fruit anthracnose in Guizhou, China. *Frontiers in Microbiology,* 13, 923181.

[29] Priwiratama, H., Wiyono, S., Hidayat, S. H., Wening, S. & Tondok, E. T. 2024a. Identification and characterization of *Curvularia*, the causal agent of leaf spot disease of oil palm seedlings in Indonesia. *Journal of the Saudi Society of Agricultural Sciences* - in press.

[30] Priwiratama, H., Wiyono, S., Tondok, E. T., Hidayat, S. H., Wening, S., Wijayanti, E. & Rozziansha, T. A. P. 2024b. Genus *Curvularia* and *Pestalotiopsis* as the primary pathogen of leaf spot disease on oil palm seedlings throughout Indonesia. *IOP Conference Series: Earth and Environmental Science,* 1308, 012018. 14-16 March, 2023, Bali, Indonesia.

[31] Ramani, V. N., Davara, D. K., Anadani, V. P. & Detroja, A. M. 2015. Evaluation of fungicides, botanicals and biocontrol agents against banana antharcnose disease under *in vitro* condition. *International Journal of Plant Protection*, 8(2), 228-233.

[32] Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W. & Consortium, F. B. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proceedings of the National Academy of Sciences*, 109(16), 6241-6246.

[33] Shanthiyaa, V., Saravanakumar, D., Rajendran, L., Karthikeyan, G., Prabakar, K. & Raguchander, T. 2013. Use of *Chaetomium globosum* for biocontrol of potato late blight disease. *Crop Protection,* 52, 33-38.

[34] Sunpapao, A., Chairin, T. & Ito, S.-I. 2018. The biocontrol by *Streptomyces* and *Trichoderma* of leaf spot disease caused by *Curvularia oryzae* in oil palm seedlings. *Biological Control,* 123, 36-42.

[35] Susanto, A. & Prasetyo, A. E. 2013. Response of *Curvularia lunata* the causal agent of oil palm leaf spot disease to various fungicides. *Jurnal Fitopatologi Indonesia,* 9(6), 165-172.

[36] Turner, P. D. & Bull, R. 1967. *Diseases and Disorders of the Oil Palm in Malaysia*. Kuala Lumpur: Incorporated Society of Planters.

[37] Witfeld, F., Begerow, D. & Guerreiro, M. A. 2021. Improved strategies to efficiently isolate thermophilic, thermotolerant, and heat-resistant fungi from compost and soil. *Mycological Progress*, 20(3), 325-339.

[38] Xu, J. 2016. Fungal DNA barcoding. *Genome,* 59(11), 913-932.

[39] Yue, H.-M., Wang, M., Gong, W.-F. & Zhang, L.-Q. 2018. The screening and identification of the biological control fungi *Chaetomium* spp. against wheat common root rot. *FEMS Microbiology Letters,* 365(22), PMID: 30289449.

[40] Zhang, G., Wang, F., Qin, J., Wang, D., Zhang, J., Zhang, Y., Zhang, S. & Pan, H. 2013. Efficacy assessment of antifungal metabolites from *Chaetomium globosum* No.05, a new biocontrol agent, against *Setosphaeria turcica*. *Biological Control,* 64(1), 90-98.