Journal of
Current Opinion in Crop Science
Journal homepage:: www.jcocs.com

RESEARCH ARTICLE

First Report on the Fungal Pathogens Associated with Leaf Tip Blight of Oil Palm at Nursery Stage in Malaysia

Nusaibah Syd Ali*, Wan Nur Asmieda and Sharifah Aliya Syed Sagaff

Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia

Edited by:

Dr. Kaliyaperumal Ashokkumar, Ph.D.,
Cardamom Research Station,
Kerala Agricultural University,
Pampadumpara, Idukki, Kerala, India.

Reviewed by:

Dr. Nor'Aishah bt Hasan, Ph.D.,
Universiti Teknologi MARA,
Cawangan Negeri Sembilan, Malaysia.

Dr. Dr. V. Prakasam, Ph.D.,
Senior Scientist (Plant Pathology),
ICAR-Indian Institute of Rice Research,
Hyderabad, Telangana, India .

Article history:

Received: February 1, 2021
Accepted: March 5, 2021
Published: March 25, 2021

Citation:

Nusaibah, S. A., Asmieda, W. N., & Sagaff, S. A. S. (2021). First Report on the Fungal Pathogens Associated with Leaf Tip Blight of Oil Palm at Nursery Stage in Malaysia. *Journal of Current Opinion in Crop Science, 2*(1), 27-39.

ABSTRACT

Oil palm has been contributing to the economic growth and gross national income (GNI) of Malaysia. On that note, supply of disease-free oil palm seedlings to field is crucial. At present many nurseries encounter disease infestations of the seedlings at nursery stages. One of the most common diseases identified was leaf tip blight. The disease incidences cause critical quality, quantity loss of the seedlings and draw other pathogens in the field to infect the initially infected host. Till to date, no study has been conducted to diagnose leaf tip blight disease of oil palm at nursery stage in Malaysia. Molecular identification was conducted by using 3 specific primers namely actin gene (ACT), β-tubulin-2 (Bt2), glyceraldehyde-3-phosphate dehydrogenase (GD) gene and complete rDNA-ITS (ITS) to confirm the isolates. All the isolated fungi matched 98% to 100% similarity with GenBank database. Thus, the isolated isolates were identified as *Nigrospora* sp*., Colletotrichum gloeosporioides* and *Phoma* sp*. In vitro* pathogenicity test demonstrated that, the three-isolates produced similar leaf tip blight symptoms as the infested seedlings at the nursery. As a conclusion, *Nigrospora* sp*., C. gloeosporioides* and *Phoma* sp. were identified as the causal pathogen of leaf tip blight of oil palm with different levels of virulence.

Keywords: Fungal pathogens; Leaf tip blight; Oil palm; *Nigrospora* sp.; *Colletotrichum gloeosporioides; Phoma* sp.

*Corresponding author e-mail address: nusaibah@upm.edu.my (S. A. Nusaibah)

INTRODUCTION

Elaeis guineensis Jacq., is originated from Africa and belongs to Arecaceae family. This tropical crop is largely cultivated for the production of vegetable oil. Malaysia and Indonesia are known to produce 80% of world's palm oil, with 39% contribution by Malaysia (Alam et al., 2015; FAO, 2016). In 2014, Malaysia has earned more than USD $22.31billion from the oil palm industry contributing to an increase in economic growth and also Gross National Income of the country (Awalludin et al., 2015).

Unfortunately, as the oil palm industry continues to expand, it is hampered with disease infestations which results in tremendous yield losses. The cultivars utilized at present are prone to varies type of diseases mainly caused by pathogenic fungi in all stages of growth. The infection from fungi may cause detrimental effects to the plant by hindering the growth rate and reduce the yield. Subsequently, prolonged infection may decline the productivity and the industry of oil palm. It has been proclaimed from most of the oil palm producing countries that there are several common diseases of oil palm. One of the most devastating oil palm disease is basal stem rot (BSR) caused by fungal pathogen identified as *Ganoderma* sp. This disease is reported as the most severe disease in both Malaysian and Indonesian oil palm plantations. This devastating disease results in direct palm loss, reduced yield and required earlier replanting which cost the planters (Flood et al., 2002; Susanto et al., 2005).

Besides that, there are also disease occurrences at nursery stage of oil palm. Through research, leaf diseases, genetic disorders and nutrient deficiency are the major constrains identified at nursery stage (Pornsuriya et al., 2013). Common serious leaf diseases at nursery stage are leaf spot and leaf blight which infest the young oil palm seedlings (Tan, 2011; Pornsuriya et al., 2013). Oil palm seedlings at nursery stages are immature and do not possess strong defense mechanism to counter pathogenic fungal infections. It has been revealed that, the major causal pathogens of leaf blight disease are a wide range of fungi (Elliot & Monica, 2009). In most cases, symptoms of leaf blight and leaf spot diseases could scarcely distinguished because it appeared to be most alike (Elliott, 2005; Kittimorakul et al., 2013). Typically, leaf blight disease symptoms will be visible on three months old oil palm seedlings (Turner, 1981). According to Kittimorakul et al. (2013), symptoms of leaf blight observed in oil palm nurseries in Southern Thailand were appearance of small circular and translucent yellow to brown necrotic tissue dispersed on the leaves. Basically, the disease symptoms will be referred to as a spot if the spots were separately located within the healthy green tissues. However, if the spots enlarged and merged or coalesced forming a massive mass of diseased tissue, the term is then referred as leaf blight (Monica and Elliott, 2015). In severe cases, the entire leaf will dried up and wilt. Besides that, there are confusions occurred between the symptoms of leaf blight disease and the manganese deficiency (Monica and Elliott, 2015). Both occur on young oil palm seedling leaves but manganese deficiency normally observed at the leaf base while leaf blight is more severe at leaf tip (Palm symptoms, 2015).

The current study was conducted to identify the symptoms and determine the causal pathogens of leaf tip blight disease of oil palm seedlings at nursery stage. This disease not only reduces the quality of oil palm seedlings, eventually it could make the seedlings more susceptible to other various diseases when it comes to transplant into the field. Therefore, it is very important to have a good and high-quality oil palm planting materials from the beginning of the cultivation to ensure the establishment of the crop. Oil palm seedlings with high resistant against pests and diseases could reduce the probability of disease occurrence at all stages including nursery and field.

In order to get a superior quality seedling with high resistance potential, intense care must be implemented in the nursery practices by following the correct method of disease management. Awareness on the disease incidence by regular monitoring the occurrence of disease symptoms is vital in order to prevent yield loss and maintain the production of healthy oil palm seedlings for the oil palm industry. To date there are only a few studies have been conducted on leaf blight disease distribution in Thailand; however, none has been documented conducted in Malaysia. Therefore, this study was conducted with the objectives (1) to isolate the causal pathogen from symptomatic oil palm seedling leaves and (2) to identify the isolated pathogen based on cultural, morphological and molecular tools.

MATERIALS AND METHODS

Sampling of Symptomatic Oil Palm Tissues

Samples of symptomatic oil palm seedling leaves with the signs of leaf blight disease were collected from nurseries in Selangor (3.0738° N, 101.5183° E) and Pahang (3.8126° N, 103.3256° E) states of Malaysia in the year 2017. The leaves were harvested and kept in a clean plastic zip lock bag at the sampling site. The samples were then brought

back to the laboratory and subjected to fungal isolation process on the same day itself. All the procedures were conducted at Biological Control Lab, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia.

Isolation of Fungi from Symptomatic Oil Palm Leaves

Isolation of fungal pathogens was conducted under aseptic conditions on four months old oil palm seedling leaves demonstrating leaf blight disease symptoms. The sterilization technique applied on the symptomatic leaf samples following the standard method by Rungjindamai et al. (2008). Samples were then cut into 4 fragments and surface sterilized by immersing them into 3% sodium hypochlorite solution for two minutes and followed by immersing it into rinsing in sterile distilled water twice. Then, the samples were tap between in sterilize filter paper to absorb the moisture and placed onto PDA plates. After three to four days of incubation at 28°C, the number of fragments incubated on PDA exhibited fungal growth. Hyphal tips of each morphologically different mycelium emerging from these tissue fragments were subcultured onto new PDA plates until pure cultures were obtained.

Characterization of Isolated Fungi

Once the pure cultures were obtained, the isolated fungal isolates were subjected to cultural and morphological characterisation.

Cultural Identification

Cultural identification is the visible observation of the fungal colonies growing on the PDA medium on day 10. The visible macroscopic features such as pigmentation, margin, elevation, colony appearances, mycelium texture and colour of upper and bottom side of the colonies were recorded.

Morphological Identification

The fungal isolates characterised culturally were then subjected to morphological characterization by observing microscopic features such as shape of conidia, presence of septate hyphae and other microscopic features via light microscope (Olympus Biological Microscope CX31). One drop of lactophenol cotton blue (LCB) was placed on a clean glass slide before transferring a small mass of aerial mycelium. Sterile transferring needle was used to spread the mycelium on the slide to allow stain penetrated the mycelium. Then, a cover slip was slowly placed to avoid air bubble formation and viewed under light microscope at magnification of ×10, ×40, and ×100. The morphological characteristics and appearances of the fungal isolates were observed and recorded.

Molecular Identification

Ten days old uncontaminated fungal isolates grown on PDA plates were used for DNA extraction. Fungal DNA extraction was conducted according to Nusaibah et al. (2011). DNA was isolated from powdered fungal mycelia (20-25mg) prepared by grinding with liquid nitrogen and extracted following Qiagen DNeasy Plant kit protocols.

Polymerase Chain Reaction (PCR)

Amplification of fungal genomic DNA was performed according to the protocols of Qiagen® TopTaq Master Mix Kit. Eppendorf Mastercycler ep Gradient S Thermal Cycler (Eppendorf, Hamburg, Germany) was used to run PCR. The PCR started with denaturation for two minutes at 95^{o}C. This was followed by 35 cycles of denaturation for one minute at 94^{o}C, annealing for 30s and temperature based on the primer set utilized and extension for two minutes at 72^{o}C. The final step of extension was carried out for ten minutes at 72^{o}C, before it was maintained at 4^{o}C. As for the negative control, sterilized distilled water was used as the DNA template instead of fungal genomic DNA. Primer sets used were, partial actin (ACT512F- 5' -ATGTGCAAGGCCGGTTTCGC -3' and ACT783R- 5' -TACGAGTCCTTCTGGCCCAT -3'), glyceraldehyde-3-phosphate dehydrogenase gene (GDF1- 5' -GCCGTCAACGACCCCTTCATTGA -3' and GDR1- 5' -GGGTGGAGTCGTACTTGAGCATGT -3'), β-tubulin (Bt2a - 5' -GGTAACCAAATCGGTGCTGCTTTC -3' and Bt2b - 5' -ACCCTCAGTGTAGTGACCCTTGGC -3') and rDNA-ITS (ITS 1 - 5' -CTTGGTCATTTAGAGGAAGTAA -3' and ITS 4 - 5' -TCCTCCGCTTATTGATATGC -3').

DNA Sequencing

DNA sequencing for forward and reverse primer of PCR amplified products were done by purifying the amplified PCR products using QIAquick Gel Extraction Kit (QIAGEN, Germany). The sequencing service was outsourced to a commercial service provider (First BASE Laboratories Sdn. Bhd. Malaysia). The sequence similarity was matched via Basic Local Alignment Search Tool (BLASTn) against the non-redundant nucleotide database in the GenBank for sequence identification purpose.

Phylogenetic Analysis

Phylogenetic tree was constructed via maximum parsimony analysis based on combined ITS, ACT, β-tubulin and GPDH nucleotide sequences using MEGA 6 software (Tamura et al., 2013) from the aligned

DNA sequences of all isolates to confirm which genera the isolates. The phylogenetic analysis showed relationships between the fungal isolates isolated from the current study with the fungal sequences cited from GenBank.

In Vitro Inoculation to Assess Pathogenicity

Oil palm leaflets harvested from healthy five months old oil palm seedlings were used for *in vitro* inoculations. Three isolated and identified fungi from symptomatic samples namely *Nigrosopra* sp. (G5), *C. gloeosporioides* (G3) and *Phoma* sp. (G2) were subjected to *in vitro* pathogenicity test. These fungal isolates were subcultured on PDA and incubated at room temperature (26±2°C) for ten days prior to inoculation. The sterilized oil palm leaves were placed onto water agar prepared on Petri dishes. A micropipette was used to place 30 µl of mycelial suspension onto the tip of the leaflet with five independent replicates. Control treatments leaves were treated with 30 µl sterilized distilled water. Petri dishes containing the treated leaflet were individually sealed with parafilm and incubated under artificial light (10 µmol m^{-2} s^{-2} provided by cool white fluorescent lamps) on a laboratory bench at 26±2°C. After three days of incubation, the spread of the advancing lesions from the point of inoculation was measured on each leaflet using a ruler. Data were arcsine transformed and analysed using ANOVA. The causal pathogens were re-isolated from the margins of lesions onto PDA. A disease severity (DS) scale was developed in the current study to assess symptoms of the disease according to the original host foliar symptoms prior to isolation. DS was scored based on the severity scale in Figure 1 and expressed according to the formula given: DS (%) = Σ (Number of seedlings in the scale × Severity scale) × 100/ Total number of seedlings assessed × Highest scale.

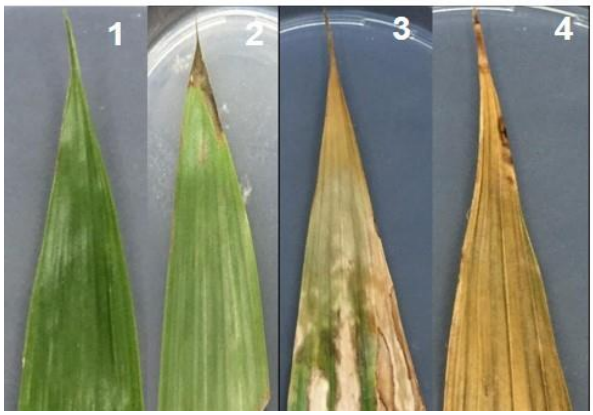

Figure 1. Scale for disease severity of leaf blight on oil palm seedlings at nursery stage. From left, (1) healthy leaf, (2) initial infection of tip leaf blight disease, (3) lesion disperse around leaf; causing blight symptoms (4) disease progressed to entire leaf; causing yellowing of the tissue, necrotic and eventually death. The severity of the leaves was taken from the original host prior to isolation step.

RESULTS

Symptoms of Tip Leaf Blight Disease on Leaves of Oil Palm Seedling

Samples of oil palm seedling leaves collected demonstrated symptoms of blight disease, which begins from the tip of the leaf. The symptoms appeared to turn the colour of the leaves from green to necrotic brown. In addition, the presence of black spores was also observed on the leaf surface (Figure 2).

Cultural and Morphological Identification

A total number of 13 fungal colonies were isolated from the symptomatic oil palm leaves, sampled from Selangor (8 isolates) and Pahang (5 isolates) nurseries. These isolates were divided into five groups; G1, G2, G3, G4, G5 and classified based on the appearance of colonies exhibited after 10 days cultured on PDA. The cultural and morphological characteristics of mycelia colonies and the formation of spores were observed on the pure cultures obtained. Through observation, each isolate on PDA medium exhibited different growth patterns and varied in growth rates. All the fungal cultures required more than a week for the mycelia to fully cover the Petri dish after incubation at temperature ±28° in the laboratory. G5 isolate required excess light for rapid mycelia formation on PDA plates compared to other 4 isolates. Observation on the cultural and morphological characterisation of the isolates was made after 10 days of incubation period (Figure 3).

The G1 isolate varied from white to grey on PDA, with dense white aerial mycelia, margin plumose. The bottom side of the colonies was rust coloured in the centre and achromatic in the edge. The colonies were then turned to black colour when older, floccose. Isolate G2 produced white to pale pink downy colonies with dense aerial mycelia. Margin even and the colonies are fealty to floccose with raised mycelia formation. The bottom side of the colonies showed 1-2 red concentric ring formed toward the edge of Petri dish. A few pale red conidial masses were observed near the inoculum.

The culture produced by G3 isolate varied from white to dark grey on PDA with dense grey aerial mycelia near the inoculum and submerged, margin irregular. The bottom side of the colonies was white with dark grey in the centre. Isolate G4 demonstrated white floccose (cottony) colonies with well-developed aerial mycelia and small black granules in the centre around the inoculum. Margin even. The bottom side of the colonies was white to pale orange in colour with achromatic edge. Formations of black spot were observed when older.

Isolate G5 produced white cottony colonies with mycelia scattered around inoculum. Margin irregular and the formation of mycelia were flat. The bottom side of the colonies was white to transparent. Formations of black spot were observed when older. The isolates were observed using light microscope to identify the microscopic features as described in Table 1. By way of contrast, G1 and G5 isolates showed different cultural morphology on plate. However, both of these isolates (G1 and G5) were identified as *Nigrospora* sp. Similarly, both G3 and G4 isolates were identified as *C. gloeosporioides* and G2 isolate was identified as *Phoma* sp.

Figure 2. Sample of infected oil palm leaf that shows symptom of leaf blight disease, (A) Black spores (in red circle) observed on the disease leaf, (B) infected oil palm leaf with fully develop lesions

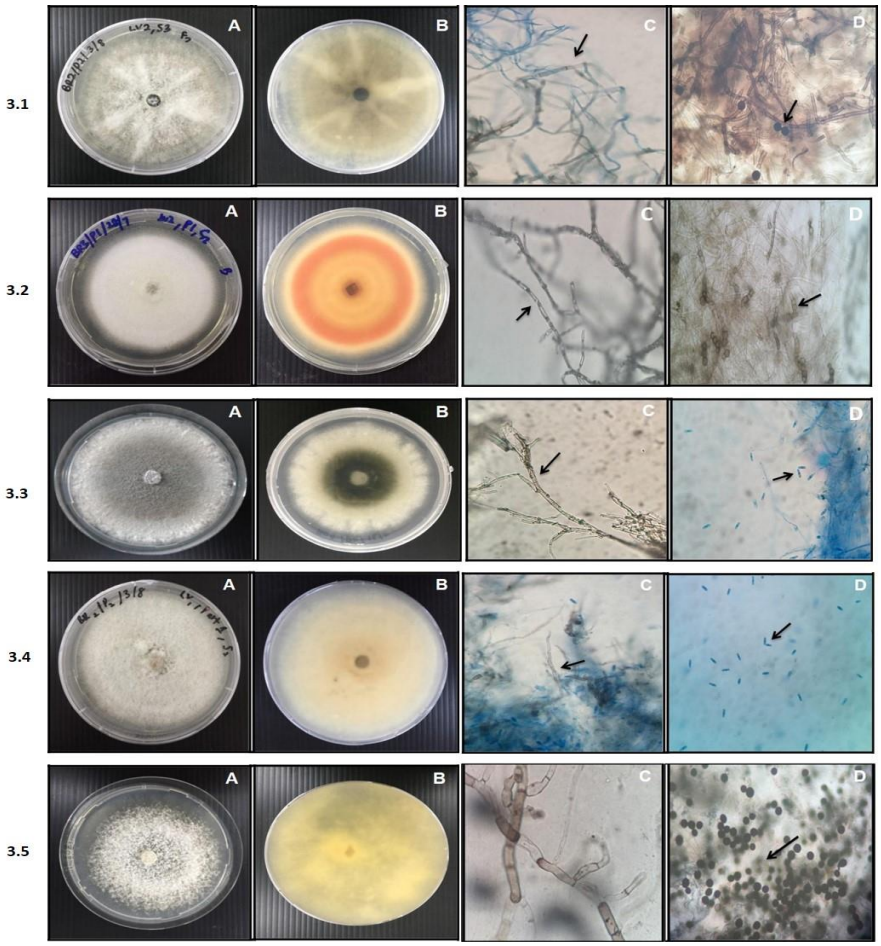

Figure 3. Colony morphology of G1-G5 isolate from infected oil palm leaf on potato dextrose agar medium (PDA); 3.1 (G1 isolate); 3.2 (G2 isolate); 3.3 (G3 isolate); 3.4 (G4 isolate); 3.5 (G5 isolate); (A) Top view of mycelium colony; (B) Bottom view of colony; (C) fungal mycelia with septate hyphae (black arrow) under light microscope (100x magnifications), (D) Conidia (black arrow) of fungal isolates under light microscope (100x magnifications)

Table 1. Morphological characteristics of isolated fungi

Isolates	Presence of septate hyphae	Presence of conidiospores	Conidia shape
G1	+	+	- Spherical and solitary - Black and opaque
G2	+	+	- Longitudinal and transverse septation within the spore.
G3	+	+	- Straight - Cylindrical - Obtuse to slightly rounded end
G4	+	+	- Straight - Cylindrical - Obtuse to slightly rounded end
G5	+	+	- Spherical and solitary - Black and opaque

Molecular Identification of the Isolated Fungal Pathogens from Symptomatic Oil Palm Leaf

The amplified PCR products of the isolates were subjected to agarose gel electrophoresis and bands were visualized after staining with EtBr. The PCR product sizes varied from 500 to 600 bp. No band was detected in the control samples (without DNA template), which indicated no contamination occurred during the PCR steps. The identification of isolates was done via sequence comparison using Basic Local Alignment Search Tool (BLASTn) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov). Fungi nucleotide sequences of partial actin gene (ACT), β-tubulin-2 (Bt2a), glyceraldehyde-3-phosphate dehydrogenase (GDF1) gene and the complete rDNA-ITS (ITS), were used to match with sequences from the most closely related organisms deposited in the GenBank database (Table 2). The result from the analysis showed that, DNA sequences of all the fungal isolates demonstrated 99 to 100% homology with the databases from GenBank. Hence, G1 and G5 isolates were identified as *Nigrospora* sp. that displayed 100% similarity. While, G2 isolates was identified as *Dothideomycetes* sp. or now known as *Phoma* sp. with 99% similarity. Lastly, G3 and G4 isolates were identified as *C. gloeosporioides* with 100% similarity. A phylogenetic tree was constructed using MEGA 6 software (Tamura et al., 2013) from the combined DNA nucleotide sequences of all isolates (Figure 4). The phylogenetic analysis showed relationships between the fungal isolates isolated from the current study with the fungal sequences cited from GenBank database. All the fungi isolates were grouped into three different clusters.

Table 2. Sequences utilized to perform phylogenetic analysis

Strain codes	Species	GenBank accession number			
		ITS	ACT512F	Bt2a	GDF1
G1	*Nigrospora* sp.	KM513608. 1	MH003698.1	KY019613.1	-
G2	*Phoma phinodella*	KX443631. 1	FJ426957.1	AY831511.1	DQ525739. 1
G3	*Colletotrichum gloeosporioides*	KM520010. 1	HQ846668.1	KM244757.1	HQ022565. 1
G4	*Colletotrichum gloeosporioides*	KR708966. 1	KF712382.1	KM244757.1	HQ022565. 1
G5	*Nigrospora* sp.	FJ527872.1	-	KY019594. 1	-

ITS-complete rDNA-ITS region, ACT actin gene- ACT512F, Bt2a-β-tubulin gene, GDF1-glyceraldehyde-3-phospahate dehydrogenase (GP

Table 3. Morphological characteristic of fungi isolated from *in vitro* pathogenicity test samples

Isolates	Presence of septate hyphae	Presence of conidiospores	Conidia shape
G2	+	+	- Longitudinal and transverse septation within the spore - *Alternaria spp.* like spores
G3	+	+	- Straight - Cylindrical - Obtuse to slightly rounded end
G5	+	-	- Spherical and solitary - Black and opaque

Table 4. Mean lesion length developed on oil palm leaflets inoculated with *Phoma* sp., *Colletotrichum gleosporioides*, and *Nigrospora* sp. after three days of inoculation at 25±1.5°C.

Isolate	Mean of lesion lenght (cm/day)	Type of fungus	Host
G5	5.18±1.25^{a}	*Nigrospora* sp.	Oil palm
G2	3.93±2.04^{ab}	*Phoma* sp.	Oil palm
G3	1.88±3.29^{b}	*Colletotrichum gleosporioides*	Oil palm

Means within columns with the same letters are not significantly different by Tukey's studentized range (HSD) test at $p \leq 0.05$. Means of lesions were calculated with five independent replicates of oil palm leaflets

Table 5. Disease severity scored based on the developed severity scale (Figure 1) on oil palm leaf inoculated with *Phoma* sp., *Colletotrichum gloesporioides* and *Nigrospora* sp. on day three

Isolate	Disease severity (%) on oil palm leaf			
	Scale 1	Scale 2	Scale 3	Scale 4
Colletotrichum gloesporioides	-	-	37.5%	-
Phoma sp.	-	-	37.5%	-
Nigrospora sp.	-	-	56.25%	-

In Vitro Pathogenicity Test

Three out of five fungi isolate namely G2- *Phoma* sp., G3- *C. gloeosporioides* and G5- *Nigrospora* sp were selected to perform *in vitro* pathogenicity test on healthy oil palm seedling leaves based on their rapid growth of PDA medium. Observation from the 72 hours incubation period exhibited development of symptoms on the inoculated oil palm leaflets. The symptoms noted were similar as the original symptoms observed on the oil palm seedling leaves at the sampling site (Figure 5). The morphological characteristics of fungi isolated from *in vitro* pathogenicity test were tabulated in Table 3. The lengths of lesions that progressed from the leaf tip of the inoculated leaves were measured after 72 hours of incubation period. Length lesion data were statistically analysed using SAS program and the mean lesion length developed were tabulated in Table 4. In order to confirm the pathogenicity of the fungal isolates used, the mycelia of the pathogens that emerged were re-isolated from the symptomatic leaves. Figure 6 displays the cultural and morphological features of the re-isolated fungal pathogens. The isolates of *Nigrospora* sp. and *C. gleosporioides* were confirmed to be the identical as the original pure culture based on the cultural and morphological appearances. However, there was a slight difference between the colours of the colony produced by isolate of *Phoma* sp. from this test compared to the original pure culture used for inoculation purpose. Disease severity scored was calculated (Table 5) based on the developed severity scale (Figure 1). The highest DS was observed on day

three after inoculation at Scale 3 (Figure 1) for all the three fungi with the percentage; *Phoma* sp. (37.5%), *C. gloesporioides* (37.5%) and *Nigrospora* sp. (56.25%).

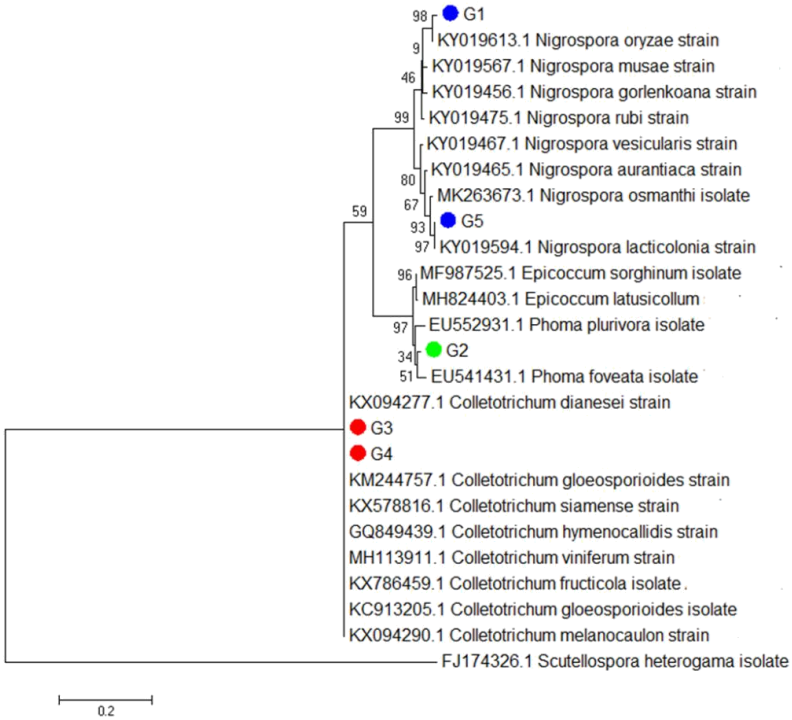

Figure 4. Phylogenetic tree constructed using MEGA 6 based on combined ITS, ACT, β-tubulin and GPDH nucleotide sequences of the five fungal isolates isolated from symptomatic oil palm leaf samples compared to fungal sequences deposited in GenBank database. Numbers at branches indicate bootstrap values (%) for 1000 replications.

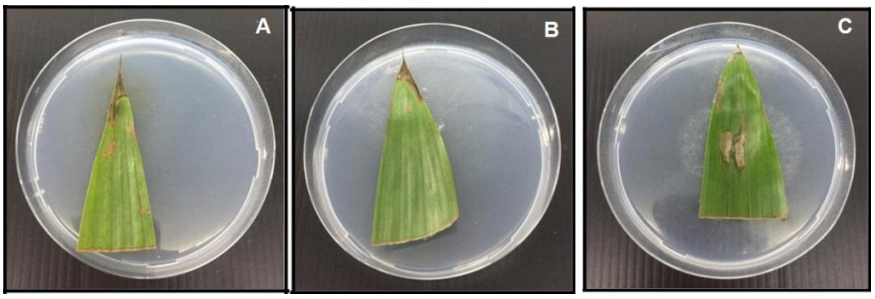

Figure 5. Lesion progress on oil palm leaflet after three days of incubation with selected fungal pathogens. A) Inoculated with *Phoma* sp., B) Inoculated with *Colletotrichum gloeosporioides*, C) Inoculated with *Nigrospora* sp.

Cultural Characteristics

The re-isolated fungal isolates from *in-vitro* pathogenicity test were subjected to cultural identification to reconfirm the identity of the initially inoculated fungal isolates as the causal pathogen. *Phoma* sp. (G2) culture produced white to pale orange downy colonies with dense aerial mycelia and even margin. The colonies are floccose with raised mycelia formation. The bottom side of the culture demonstrated pale orange concentric rings near the inoculum. There were also black mycelia formations around the inoculum. *C. gloesporioides* (G3) culture produced white to grey aerial mycelia near the inoculum with even. Bottom side of the culture showed two to three dark grey concentric rings. *Nigrospora* sp. (G5) culture produced white cottony colonies with mycelia scattered around inoculum with irregular margin. The bottom side of the colonies was white to transparent.

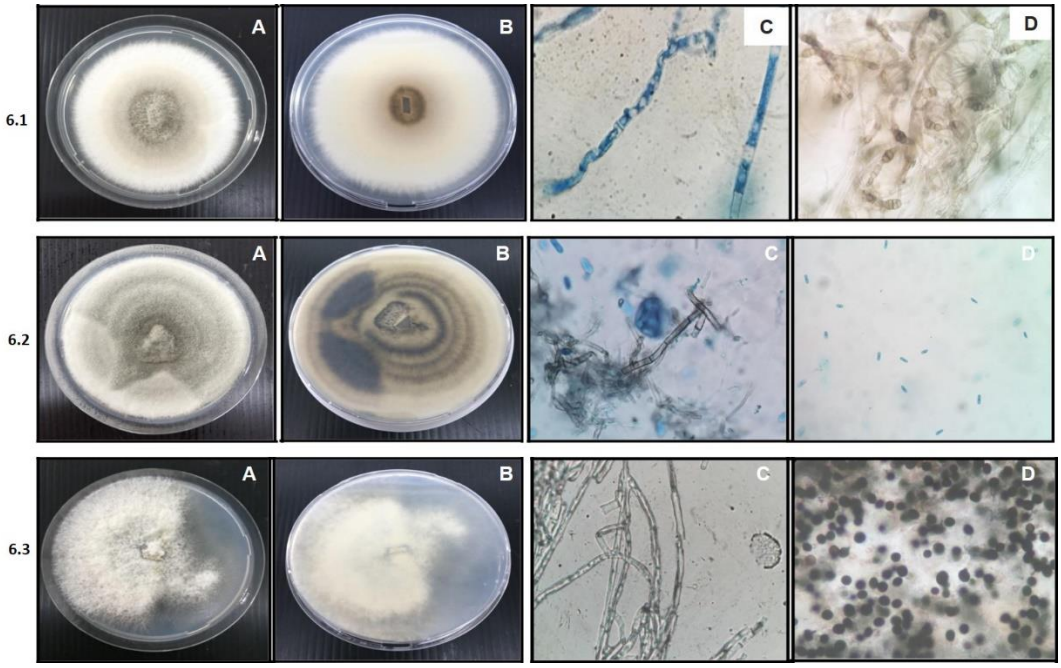

Figure 6. Cultural and morphological features of identified isolates from symptomatic oil palm leaf after six-day incubations period in *in vitro* pathogenicity test; 6.1 (*Phoma* sp.); 6.2 (*Colletotrichum gloesporioides*); *6.3* (*Nigrospora* sp.); (A) Top view of mycelium colony, (B) bottom view of colony, (C) fungal mycelia with septate hyphae under light microscope (100x magnifications), (D) Conidia of fungal isolates under light microscope (100x magnifications)

DISCUSSIONS

Symptoms of Leaf Blight Disease on Oil Palm Seedlings and Isolation of the Causal Agent

Field evaluation and sample collection are vital in order to determine the causal pathogen of leaf tip blight disease. The symptoms observed on the oil palm seedling leaf samples collected from nurseries in Pahang and Selangor indicated that the oil palm seedlings were infected with leaf disease identified as leaf tip blight. The initial symptom begins from the leaf tip and visible dark spots scattered individually on the leaf surface. There is also yellowish-brown zone encircle the spots and over time the diameter of the zone will be enlarged from round shape producing oval lesions on the infected leaves. The lesions produced were irregular in shape, large and coalesced causing the diseased leaves to change colour from green to brown necrotic and becomes fully blighted. Eventually, the blighted leaves will die due to the disease progress. Kittimorakul et al. (2013) described that the symptoms of leaf blight disease were characterized as small disc-shaped and transparent yellowish-brown necrotic tissues disseminated on the surface of the infected leaves. In addition, black in colour sporulation were detected on the samples collected where this area was used for the isolation purpose in this study.

Cultural and Morphological Characterisation of Potential Fungal Pathogens

Nigrospora sp. isolates; G1 and G5 produced colony colours of white to grey at initial stages of incubation on PDA and black in colour as it aged due to heavy sporulation. These observations were in agreement with the previous studies (Shekhawat et al., 2010; Abass & Mohammed, 2014; Zarandi et al., 2014). Similar findings by Rai et al. (2014) reported that fungi from genus *Phoma* reproduced asexually and the colony formed by this genus depends on the species which commonly have velvety surface and varied from white to grey with reddish purple and pink colourations. Seredich (2016) discovered that, the formation process of vegetative resting spores of *Phoma* sp. which was initially the protoplasm fragments will be segregated by septation of mycelium producing chlamydospores. The morphological characteristics observed from G2 isolates in this study were homogenous with the generic principle of *Phoma* sp. as reported by Rai et al. (2014) and Seredich (2016). Despite that, the conidia of *Phoma* in this study contradicted with the conidia described by Panja et al. (2016). The shape of conidia

reported by Panja et al. (2016) was single-cell without any septation, elongated, very warty, eguttulate and the tip of spores were rounded. Though, in this study the conidia have more than one septation within the spore and not warty. Thus, it can be highlighted that the morphological description of *Phoma* sp. isolated from symptomatic oil palm seedling leaves in this present study was flagrantly distinct from *Phoma* sp. that causes leaf tip blight disease on tuberose described by Panja et al. (2016). The colony colour of G3 isolate was identical with the one reported by Kittimorakul et al. (2013), and the colony of G4 isolates was alike with the work of Photita et al. (2005). Despite having divergence on the colour of colony produced, both isolates showed similar microscopic characteristics where the conidia present are elliptical in shape. Therefore, both of the isolates were identified as *C. gloeosporioide.* Sonoda and Pelosi (1988) mentioned that there are two types of *C. gloeosporioides* culture on plate; the first is a fast growing and grey in colour while the second is the slow-growing type with orange colour colonies. This literature review matches up with the types of *C. gloeosporioides* colonies obtained in this current study. The differences in the morphological traits exhibited by fungal pathogens are due to the environmental factors which influence the stability of the existing morphological form (MacLean et al., 1993; Cannon et al., 2000).

Molecular Identification

A more accurate and sensitive diagnostic tool which is the molecular method were employed in this recent study to support the cultural and morphological identification carried out. Molecular method is basically based on *in vitro* amplification of fungal DNA using PCR technology (Moricca et al., 1998). Analysis from the phylogenetic tree showed that isolate G1 and G5 were classified together in the same clade with the sequences of *Nigrospora* sp. including *N. sphaerica* and *N. oryzea* with 99% of bootstrap value. Besides that, the second cluster in the phylogenetic tree with isolate G2 was divided into two sub-clusters. Previous studies emphasized that *Dothideomycetes* sp. could also be identified as *Phoma* sp. (Kirk et al., 2008; Crous et al., 2013). It is the largest and most arguable in terms of phylogenetic diversity class within the fungal phylum Ascomycota (Kirk et al., 2008; Crous et al., 2013). On that account, isolate G2 was determined as *Phoma* sp. because the analysis could not precisely identify the species it belongs to. Meanwhile, isolate G3 and G4 were grouped together in the same clade with *C. gloeosporioides* with high support of bootstrap value 100%. The molecular method has been successfully defeating the limitation of traditional method in the identification and differentiating phytopathogenic fungi species and in describing the species (Sreenivasaprasad et al., 1996; Johnston & Jones, 1997). Furthermore, the analysis using actin gene (ACT), β-tubulin-2 (Bt2a), glyceraldehyde-3-phosphate dehydrogenase (GDF1) genes and the complete rDNA-ITS (ITS) gave adequate information to conclude phylogenetic relationships among the isolated fungal pathogens (Sreenivasaprasad et al., 1996; Freeman et al., 2000). Hence, based on both morphological and molecular characters of isolated fungi, it has been confirmed that the identity of causal agents of leaf blight disease on oil palm seedlings in nurseries of Pahang and Selangor were identified as *Nigrospora* sp., *Phoma* sp., and *Collectotrichum gloeosporioides.*

In Vitro Pathogenicity Test

In vitro pathogenicity test conducted in this study confirmed that *Nigrospora* sp., *C. gloeosporioides* and *Phoma* sp. were the causal agents that caused leaf tip blight on oil palm seedlings based on the lesion progressed on the inoculated oil palm leaflets. The symptoms exhibited on the inoculated oil palm leaflets were similar with the original symptom observed. First visible symptom was a small dark spot at the tip of the leaflet and later became lesions, which elongated and coalesced as the disease developed. Eventually, the colour of the infected area on the leaflets turned from green to brown colour. Over time, black sporulation was observed formed on the dead tissues of the leaflets. Identical symptoms with the current study were reported by Panja et al. (2016) on the tuberose leaf infected with leaf tip blight disease caused by *Phoma mondouriensis*. The isolated fungal pathogens from the inoculated oil palm leaves showed similar morphological characteristic with the previous isolated culture except for one pathogen. Both *C. gloeosporioides* and *Nigrospora* sp. cultures re-isolated from this test expressed similar cultural characteristics with the original cultures and only *Phoma* sp. re-isolated gave different cultural appearance.

Phoma culture derived from this test showed different cultural appearance where the colour of colony produced on PDA medium varied from whitish-orange to dark grey. As a comparison, the previous culture was white to pale pink towards the inoculum and did not comprise mycelia with dark grey in colour. Based on the morphological identification carried out, similar pattern of hyphae and conidia features were obtained. This contradiction in the colony features could be due to the environmental conditions during the incubation

step. This is because the previous *Phoma* culture was isolated during rainy season while the present *Phoma* culture was isolated during hot season. According to Rai et al. (2014), environmental factors such as lighting, temperature, and pH will influence the stability and appearances of the morphological and cultural traits of *Phoma* species.

Statistical analysis demonstrated that, there were significant differences between the mean length lesion produced by *Nigrospora* sp. (G5) with *Phoma* sp. (G2) and *C. gloeosporioides* (G3). However, there are no significant differences between the mean length lesion produced *Phoma* sp. (G2) and *C. gloeosporioides* (G3). *Nigrospora* sp. (G5) exhibited the longest lesion length on the oil palm seedling leaflets while the lowest lesion length was produced by *C. gloeosporioides* (G3). Fungi from genus *Nigrospora* are often found to be an opportunistic pathogen or saprophyte on diverse type of plant species and also as causal pathogen of leaf disease on a wide range of plant hosts (Dutta et al., 2015; Wright et al., 2008). It had been reported that *Nigrospora sphaerica* as the foliar pathogen responsible to cause leaf blight disease on tea in India (Dutta et al., 2015). In addition, Wright et al. (2008) discovered that *N. sphaerica* is the secondary pathogen that causes leaf spot and twig and shoot blight on blueberry that the mechanism of infection is through the wounds made by insects. Besides that, *Nigrospora oryzae* was reported as a crucial fungal pathogen that causes leaf spot on *Aloe vera* in China, Iraq and Pakistan (Zhai et al., 2013; Alam et al., 2017). Recently, *N. sphaerica* has been reported for to cause leaf blight disease on Chinese fir, the timber tree in China (Xu & Liu, 2017). *Phoma* spp. is a type of fungi that have high resistance towards chemical substances such as fungicide. *Phoma* spp. could persist longer in the environment which helps them to rapidly multiply in the susceptible hosts (James, 1989). This species could be found extensively in most of the ecological niches. It is known as one of the most crucial phytopathogenic fungi that may give harmful effects towards commercially important plants (Aveskamp et al., 2008; Aveskamp et al., 2010). It has been recorded that *Phoma herbarum* and *Phoma glomerata* are the pathogens responsible to cause leaf spot and ascochyta blight complex on field pea in Australia (Li et al., 2011; Tran et al., 2014). Besides that, *Phoma* sp. also has been reported for the first time by Rhouma et al. (2010) to cause branch dieback of olive tree in Tunisia. Kittimorakul et al. (2013), stated that one of the fungal pathogens that causes leaf blight disease on oil palm seedlings in Southern Thailand belongs to the genera *Collectotrichum*. *Colletotrichum* spp. are virulent plant pathogens known to infect a wide range of host plants and contributed to severe disease incidence to the infested plants (Sutton, 1980); Photita, 2005). This pathogen has been described as the causal agent to cause anthracnose disease on oil palm seedlings at nursery stage (Kittimorakul et al., 2013). Therefore, the *in vitro* pathogenicity test conducted in the present study indicated that *Nigrospora* sp., *Phoma* sp., and *C. gloeosporioides* exhibited pathogenic criterions that lead to the development of leaf tip blight symptoms on oil palm seedlings with different virulence level

CONCLUSION

As a conclusion, we could conclude that, leaf tip blight disease can be considered as a complex disease that may require more than one fungal pathogen. In addition, to further confirm the complexity of this disease, further research via *in vivo* pathogenicity test of the identified fungal pathogens would be vital.

ACKNOWLEDGEMENT

This project was supported by the Fundamental Research Grant Scheme (FRGS), administered through the Ministry of Higher Education, Malaysia (Grant No: FRGS/1/2018/WAB01/UPM/02/31/5540093).

DECLARATION ON CONFLICT OF INTEREST

No conflict of interest among the authors.

AUTHOR CONTRIBUTIONS STATEMENTS

Nusaibah Syd Ali designed the experiments, collected symptomatic oil palm leaf samples, funded the project and wrote the paper. Wan Nur Asmieda carried out the experiments. Sharifah Aliya Syed Sagaff repeated one part of the experiment and formatted the paper.

REFERENCES

Abass, M. H., & Mohammed, N. H. (2014). Morphological, molecular and pathological study on *Nigrospora oryzae* and *Nigrospora sphaerica*, the leaf spot fungi of date palm. *Basra Journal for Date Palm Researches, 13*, 1-2.

Alam, A. F., Er, A. C., & Begum, H. (2015). Malaysian oil palm industry: prospect and problem. *Journal of Food, Agriculture and Environ*ment, 13(2), 143-148.

Alam, M. W., Rehman, A., Saira, M., Khan, N. A., Aslam, S., Fiaz, M., & Muhammad, S. (2017). First Report of Leaf Spots in Aloe vera Caused by *Nigrospora oryzae* in Pakistan. *Plant Disease*, *101*(5), 841.

Aveskamp, M. M., De Gruyter, J., & Crous, P. W. (2008). Biology and recent developments in the

systematics of *Phoma*, a complex genus of major quarantine significance. *Fungal Diversity, 31*, 1-18.

Aveskamp, M. M., De Gruyter, J., Woudenberg, J. H. C., Verkley, G. J. M., & Crous, P. W. (2010). Highlights of the *Didymellaceae*: A polyphasic approach to characterise *Phoma* and related pleosporalean genera. *Studies in Mycology, 65*, 1-60.

Awalludin, M. F., Sulaiman, O., Hashim, R., & Nadhari, W. N. A. W. (2015). An overview of the oil palm industry in Malaysia and its waste utilization through thermochemical conversion, specifically via liquefaction. *Renewable and Sustainable Energy Reviews, 50*, 1469-1484.

Cannon, P.F., Bridge, P.D., & Monte, E. (2000). Linking the past present and future of *Colletotrichum* systematics. In D. Prusky, S. Freeman, and M.B. Dickman (Eds.) *Colletotrichum host specificity, pathology and host pathogen interaction*. APS press, St Paul, Minnesota: 1-20.

Crous, P. W., & Gams, W. (2006). «Phaeomoniella chlamydospora» Gen. et Comb. Nov., a Causal Organism of Petri Grapevine Decline and Esca. *Phytopathologia Mediterranea, 39*(1), 112-118.

Crous, P. W., Verkley, G. J., & Groenewald, J. Z. (Ed). (2013). *Phytopathogenic Dothideomycetes*. CBS-KNAW Fungal Biodiversity Centre.

Dutta, J., Gupta, S., Thakur, D., & Handique, P. J. (2015). First report of *Nigrospora* leaf blight on tea caused by *Nigrospora sphaerica* in India. *Plant Dis*ease, *99*(3), 417.

Elliott & Monica, L. (2009). Leaf Spots and Leaf Blights of Palm 1. Disease Management. 1–4. Retrieved from http://edis.ifas.ufl.edu/pdffiles/PP/PP14200.pdf.

FAO. (2016). http://faostat3.fao.org /faostat-gateway /go/to/home/E (accessed on November 8 2017).

Flood, J., Hasan, Y. & Foster, H. (2002). Ganoderma diseases of oil palm-an interpretation from Bah Lias Research Station. *Planter, 78*(921), 689-710.

Freeman, S., Minz, D., Jurkevitch, E., Maymon, M., & Shabi, E. (2000). Molecular analyses of *Colletotrichum* species from almond and other fruits. *Phytopathology, 90*, 608–614.

James, R. L. (1989). Effects of fumigation on soil pathogens and beneficial microorganisms. General Technical Report. RM-Rocky Mountain Forest and Range Experiment Station, US Department of Agriculture Forest Service, Fort Collins, CO.

Johnston, P. R. & Jones, D. (1997). Relationships among *Colletotrichum* isolates from fruit-rots assessed using rDNA sequences. *Mycologia,* 420-430.

Kirk, P.M., Cannon, P.F., Minter, D.W. & Stalpers, J.A. (2008). *Ainsworth and Bisby's dictionary of the Fungi,* (10th ed.). CAB International, 2283pp. Wallingford: U.K.

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.

Li, Y. P., You, M. P., Khan, T. N., Finnegan, P. M. & Barbetti, M. J. (2011). First report of *Phoma herbarum* on field pea (*Pisum sativum*) in Australia. *Plant Disease*, *95*(12), 1590-1590.

MacLean, D.J., Braithwaite, K.S., Manners, J.M. & Irwing, J.A.G. (1993). How do we identify and classify fungal pathogens in the era of DNA analysis? *Advances in Plant Pathology, 10*, 207-244.

Moricca, S., Ragazzi, A., Kasuga, T. & Mitchelson, K. R. (1998). Detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue by polymerase chain reaction. *Plant Pathology, 47*(4), 486-494.

Nusaibah, S. A., Latiffah, Z., & Hassaan, A. R. (2011). ITS-PCR-RFLP analysis of *Ganoderma* sp. infecting industrial crops. *Pertanika Journal of Tropical Agricultural Science*, *34*, 83–91.

Panja, B. N., De, A., Mandal, M., & Saha, J. (2016). First report on leaf tip blight disease of tuberose (*Polyanthes tuberosa* L.) caused by new *Phoma* sp., *Phoma mondouriensis* from West Bengal. *Journal of Mycopathological Research*, *54*(1), 171-174.

Photita, W., Taylor, P. W., Ford, R., Hyde, K. D. & Lumyong, S. (2005). Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. *Fungal Diversity, 18,* 117-133.

Pornsuriya, C., Sunpapao, A., Srihanant, N., Worapattam, K., Kittimorak, J., Phithakkit, S. & Petcharat, V. (2013). A Survey of Diseases and Disorders in Oil Palms of Southern Thailand. *Plant Pathology Journal, 12*(4), 169–175. https://doi.org/10.3923/ppj.2013.169.175.

Rai, M. K., Tiwari, V. V., Irinyi, L. & Kövics, G. J. (2014). Advances in taxonomy of genus *Phoma*: polyphyletic nature and role of phenotypic traits and molecular systematics. *Indian Journal of Microbiology, 54*(2), 123-128.

Rhouma, A., Triki, M. A., Krid, S., Tuset, J. J. & Msallem, M. (2010). First report of a branch dieback of olive trees in Tunisia caused by a *Phoma* sp. *Plant Dis*ease, *94*(5), 636.

Rungjindamai, N., Pinruan, U., Choeyklin, R., Hattori, T. & Jones, E. B. G. (2008). Molecular characterization of basidiomycetous endophytes isolated from

leaves, rachis and petioles of the oil palm, *Elaeis guineensis*. Thailand. *Fungal Diversity, 33*, 139-161.
Seredich, M. O. (2016). Cultural and morphological characteristics of fungus *Phoma* sp. 1–Phoma blight causing agents in coniferous tree seedlings. *Proceedings of BSTU*, *1*, 98-101.
Shekhawat, K. K., Rao, D. V. & Batra, A. (2010). Morphological study of endophytic fungi inhabiting leaves of *Melia azedarach* L. *International Journal of Pharmaceutical Sciences Review and Research, 5*(3), 177-180.
Sonoda, R. M. & Pelosi, R. R. (1988). Characteristics of *Colletotrichum gloeosporioides* from lesions on citrus blossoms in the Indian River area of Florida. *US Department of Agriculture*, *82*, 200-205.
Sreenivasaprasad, S., Mills, P.R., Meehan, B.M. & Brown, A.E. (1996). Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. *Genome*, *38*, 499–512. DOI: 10.1139/g96-064
Susanto, A., Sudharto, P. S. & Purba, R. Y. (2005). Enhancing biological control of basal stem rot disease (*Ganoderma boninense*) in oil palm plantations. *Mycopathologia*, *159*(1), 153-157.
Sutton, B.C. (1980). The Coelomycetes. Commonwealth Mycological Institute. Kew, UK.
Tan, C. C. (2011). *Nursery Practices for Production of Superior Oil Palm Planting Materials. Agronomic Principles and Practices of Oil Palm Cultivation*. K.J., Goh, S.B. Chiu & S. Paramananthan (Ed.). Agricultural Crop Trust, Malaysia. 145-168.
Tran, H. S., You, M. P., Lanoiselet, V., Khan, T. N. & Barbetti, M. J. (2014). First report of *Phoma glomerata* associated with the ascochyta blight complex on field pea (*Pisum sativum*) in Australia. *Plant Disease, 98*(3), 427. http://dx.doi.org/10.1094/PDIS-08-13-0809-PDN
White, T. J., Bruns, T., Lee, S. J. W. T. & Taylor, J. L. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M.A. Innis, D.H. Gelfand, J.J. Sninsky, J.J. & White, T.J. (ed.). *PCR protocols: a guide to methods and applications*, pp. 315-322. Academic Press, New York.
Wright, E. R., Folgado, M., Rivera, M. C., Crelier, A., Vasquez, P. & Lopez, S. E. (2008). *Nigrospora sphaerica* causing leaf spot and twig and shoot blight on blueberry: a new host of the pathogen. *Plant Disease, 92*(1), 171.
Xu, Y. M. & Liu, Y. J. (2017). First Report of *Nigrospora sphaerica* Causing Leaf Blight on *Cunninghamia lanceolata* in China. *Plant Disease*, *101*(2), 389.
Zarandi, D. M., Aminaee, M. M., Sharzei, A. & Rezaee, S. (2014). First report of rosemary leaf spot caused by *Nigrospora oryzae* in Iran. *New Disease Reports,* 30, 27.
Zhai, L. F., Liu, J., Zhang, M. X., Hong, N., Wang, G. P. & Wang, L. P. (2013). The first report of leaf spots in *Aloe vera* caused by *Nigrospora oryzae* in China. *Plant Disease*, *97*(9), 1256. http://dx.doi.org/10.1094/PDIS-03-13-0314-PDN