Study on Endophytic Fungi Associated with *Moringa oleifera* Lam. Collected from Lombok Island, West Nusa Tenggara

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Abstract

An assemblage of endophytic fungi was isolated from *Moringa oleifera* Lam. collected from Lombok island, West Nusa Tenggara Province, Indonesia. Fungal endophytes were isolated using surface sterilization methods with slight modification. Forty-six selected endophytic fungal strains were isolated from the leaves, petioles, and stems of *M. oleifera*. The fungal strains identification through morphological observation and ITS rDNA-based molecular analysis showed that fungal endophytes were associated with host plants belonging to the taxa *Alternaria*, *Cladosporium*, *Colletotrichum* (*Glomerella*), *Corynespora*, *Curvularia* (*Cochliobolus*), *Fusarium*, *Mucor*, *Ochrocladosporium*, *Phomopsis* (*Diaporthe*), and *Trametes*. In this study, endophytic *Phomopsis* dominating the obtained strains, whereas 26% (12/46) strains were isolated from host plant samples.

Keywords: endophytic fungi, fungal identification, fungal isolation, ITS rDNA, *Moringa oleifera* Lam.

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Introduction

Moringa oleifera Lam., widely known as 'drumstick tree', is one of the 'miracle tree' because all of its parts are widely used for nutritional and medicinal purposes. This plant is known to have beneficial properties including antioxidants, antibacterial, anti-fungal, anti-cholesterol, anti-inflammatory, anti-ulcer, pain relief, immunomodulatory, and wound healing (Fuglie, 1999; Hassan & Ibrahim, 2013; Aminah *et al*., 2015). *Moringa oleifera* can associate with microorganisms that naturally live in plant tissues as beneficial symbionts and are able to live by forming colonies without endangering their hosts. These microorganisms, called endophytes, can be bacteria or fungi. However, endophytic fungi are more abundant than endophytic bacteria in nature (Tan & Zou, 2001).

Endophytic fungi could be found in every part of the plant, such as leaf, stem, root, flower, fruit, and seed. Endophytic fungi can produce secondary metabolites that depend on their host plant. Those secondary metabolites protect the host plant itself and can act as antimicrobial and antivirus. Moreover, the use of secondary metabolites of endophytes could also reduce the overexploitation of medicinal plants as the source materials of drugs and the production cost of medicines (Dhanalakhsmi *et al*., 2013; Kursia *et al*., 2018).

Exploring endophytic fungi from plants will be useful in finding endophytic fungi that possess specific and unique properties. Endophytic fungi isolated from medicinal plants are considered as an attractive source of novel bioactive compounds (Tan & Zou, 2001; Strobel *et al.*, 2004; Zhang *et al.,* 2006; Kumar *et al.,* 2014). According to Strobel and Daisy (2003), the endophytic fungi, which were isolated from *Taxus brevifolia,* have the ability to produce active compounds such as paclitaxel or taxol, which is beneficial as an anticancer agent.

Various types of plants can be used as the hosts for endophytic fungi. As mentioned above, an endophytic fungus from medicinal plants is a source of beneficial secondary metabolites. It also can produce bioactive compounds which are potential as materials for producing modern medicine or agrochemical

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application (Widowati *et al*., 2016; Praptiwi *et al*., 2018). One of the plant species which can be a host for endophytic fungi is *M. oleifera* Lam. It is a small, fast-growing, deciduous, or evergreen tree which can grow up to 10–12 m in height (Roloff *et al*., 2009). This study was conducted to investigate the abundance and diversity of endophytic fungi inhabiting *M. oleifera* collected from Lombok island, West Nusa Tenggara Province, Indonesia.

Materials and Methods

Plant Materials. The plant materials of *Moringa oleifera* was collected from Lombok island, West Nusa Tenggara Province, Indonesia, on 11 April 2019 (Table 1). Plant samples consist of fresh material and healthy living tissue of *M. oleifera* leaves, petioles, and stems. All fresh samples were marked, packed carefully, and then transferred to the laboratory for isolation purposes within less than 72 h.

Table 1. List of *Moringa oleifera* Lam. samples collected from Lombok island, West Nusa Tenggara Province

No	Sample Code	Sample Description	Sample Collector	Sampling Site	GPS Coordinate	Altitude (m.alt)
1	ML 01	Leaves, petioles, and stems of *M. oleifera*	MI & IR*	Narmada park, Lembuah village, Narmada sub regency, West Lombok regency	S 08º35'41.8" E 116º12'15.1"	121
2	ML 02	Leaves, petioles, and stems of *M. oleifera*	MI & IR*	Tragtag village, Lingsar sub regency, West Lombok regency	S 08º34'21" E 116º11'5.6"	134
3	ML 03	Leaves, petioles, and stems of *M. oleifera*	MI & IR*	Selat village, Lingsar sub regency, West Lombok regency	S 08º34'53.2" E 116º12'40.4"	174

Notes: (*) Muhammad Ilyas and Indriati Ramadhani

Fungal Isolation.

The fungal isolation was carried out based on the surface sterilization method with slight modification (Ilyas *et al.,* 2019). Plant samples were sterilized by immersing in 70% ethanol for 1 min, and then sterilized with 1% sodium hypochlorite (NaOCl) solution for 2 min. Samples were rinsed twice in sterile distilled water and put into sterile paper towels for 3-4 h to remove water from the surface. Afterward, samples aseptically cut into small segments about 5 mm^2 and then placed onto a 90-mm Petri dish containing malt extract agar (MEA). On each MEA plate, 7 small segments of leaves, 6 segments of petioles, and 5 segments of stem samples were randomly placed. Each plant sample was made as many as 3 replicates. The culture was then incubated at 27 ºC for 2 weeks. The endophytic fungi growing out from samples were isolated and purified by transferring onto a 60-mm Petri dish containing potato dextrose agar (PDA). The purified fungal strains were then selected for working and backup collections. The backup collections were stored based on a freezing method at -80ºC using 10% (v/v) glycerol and 5% (g/v) trehalose as a cryoprotectant (Kanti *et al.,* 2018).

Molecular identification.

The molecular identification was based on the DNA sequence analysis of an internal transcribed spacer (ITS1 and ITS2) of rDNA regions, including 5.8S rRNA. The fungal genomic DNA was isolated using Nucleon PhytoPure (GE Healthcare) according to the manufacturer's instruction. DNA amplification of the ITS rDNA region was performed by polymerase chain reaction (PCR). PCR amplification was performed in a 25-µL reaction mixture containing 10 µL of distilled water, 12.5 µL of GoTaq Green Master Mix (Promega), 0.5 µL of DMSO, 0.5 µL of each primer (10 pmol), and 1 µL (5-10 ng) of genomic DNA as a template. The primer set of ITS4 (5`-TCC TCC GCT TAT TGA TAT GC-3`) and ITS5 (5`-GGA AGT AAA AGT CGT AAC AAG G-3`) was used to amplify approximately 550 nucleotides from ITS1 and ITS 2 including 5.8S rDNA (White *et al.,* 1990). Amplification was performed in a TaKaRa PCR Thermal Cycler P650 (TAKARA BIO Inc.) under the following conditions: initial denaturation at 95 °C for 3 min, 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The PCR products were then subjected to purification and sequence analysis.

Data Analysis.

The occurrence of fungal endophyte in the plant sample part was measured by the colonization frequency (CF) or colonization rate (CR). CF is the number of plant segments

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with endophytic fungi colonization divided by the total number of all incubated segments (Yuan *et al*., 2010). CF in the plant part is the average CF of each plant segment. CF in the plant is the average CF of all segments from one plant sample. CF, CF in the plant part, and CF in the plant are expressed in percentage.

Meanwhile, the presence of each fungal endophyte species was measured by the isolation frequency (IF). IF is the number of plant fragments from which the fungus was isolated divided by the total number of seeded fragments (Ragazzi *et al*., 2001). IF is expressed in percentage. The results of the data obtained were then analyzed using a descriptive method.

Phylogenetic Analysis.

The raw sequence data were edited using SeqMan Pro version 7.1.0 (44.1) in the DNASTAR laser gene core suite software (DNASTAR Inc., Madison, WI, USA). The assembled sequences were aligned against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nlh.gov/) using the MUSCLE (Edgar, 2004) in MEGA version 7 program (Kumar *et al*., 2016). The phylogenetic analyses of sequence data were performed based on the Neighbor-Joining (NJ) method (Saito & Nei, 1987) using the Kimura 2-parameter + G + I model as the best evolutionary model in MEGA 7 (Kimura, 1980). The reliability of each branch was evaluated by bootstrapping with 1000 resampling in NJ (Felsenstein, 1985).

Results

Colonization and Isolation Frequency of Endophytic Fungi.

In total, 63 leaf, 54 petiole, and 45 stem segments from three plant samples of *M. oleifera* were used and analyzed in this study. The fungal endophytes started growing from plant tissues after 5-7 d incubation at 27 °C (Figure 1). The occurrence of endophytes in each plant part or segment was then calculated based on the colonization frequency (CF) equation, and the results are depicted in Table 2.

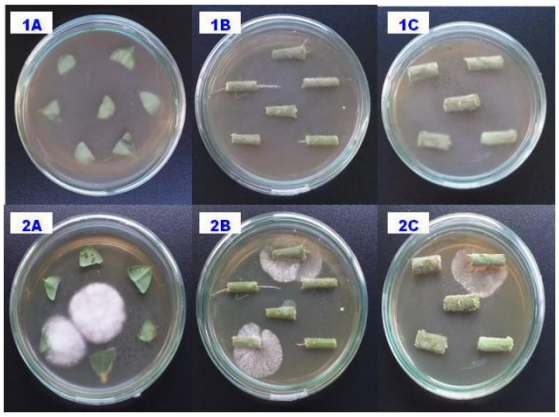

Figure 1. Fungal endophytes associated with *Moringa oleifera* Lam. isolation based on surface sterilization method: Leaves samples (1A), petioles samples (1B), and stems samples (1C). Fungal mycelia were grown from samples on MEA media after 5-7 d incubation at 27 °C (2A, 2B, 2C).

Table 2. Percentage of colonization frequency (CF) of the endophytic fungi inhabiting *Moringa oleifera* Lam. collected from Lombok island, West Nusa Tenggara Province

No.	PS	SP	SC	TCF	TSO	CF	CFPP	CFP
1.	*M. oleifera* I	L	ML1A	1	7	14.28	19.04	12.27
			ML1B	2	7	28.57		
			ML1C	1	7	14.28		
		P	MP1A	1	6	16.67	11.11	
			MP1B	1	6	16.67		
			MP1C	-	6	-		
		S	MS1A	-	5	-	6.67	
			MS1B	-	5	-		
			MS1C	1	5	20.00		
2.	*M. oleifera* II	L	ML2A	5	7	71.42	52.37	65.60
			ML2B	2	7	28.57		
			ML2C	4	7	57.14		
		P	MP2A	3	6	50.00	44.44	
			MP2B	3	6	50.00		
			MP2C	2	6	33.33		
		S	MS2A	5	5	100.00	100.00	
			MS2B	5	5	100.00		
			MS2C	5	5	100.00		
3.	*M. oleifera* III	L	ML3A	-	7	-	14.28	17.35
			ML3B	2	7	28.57		
			ML3C	1	7	14.28		
		P	MP3A	1	6	16.67	11.11	

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		MP3 B	1	6	16.67	
		MP3 C	-	6	-	
		MS3 A	1	5	20.00	
	S	MS3 B	2	5	40.00	26.67
		MS3 C	1	5	20.00	

PS plant sample, *SP* sample part, *SC* sample code, *TCF* total colony formed, *TSO* total segment observed, *CF* colonization frequency, *CFPP* colonization frequency in plant part, *CFP* colonization frequency in plant, *L* leaf, *P* petiole, *S* stem

Based on the colonization frequency (CF) results, the highest amount of endophytic fungi was from the leaves and petioles, while the least amount of endophytic fungi was from the stems part of *M. oleifera*. Further analysis of fungal endophyte occurrence based on fungal diversity in each plant parts can be seen in Table 3.

Table 3. Percentage of isolation frequency (IF) fungal endophytes inhabiting *Moringa oleifera* Lam. from Lombok Island, West Nusa Tenggara

Fungal Taxa	*M. oleifera* I			*M. oleifera* II			*M. oleifera* III		
	L	P	S	L	P	S	L	P	S
Alternaria	-	-	-	4.76%	-	-	4.76%	-	-
Cladosporium	-	5.55%	-	-	-	-	-	-	-
Colletotrichum (*Glomerella*)	-	-	-	4.76%	-	-	4.76%	-	-
Corynespora	-	-	-	4.76%	-	-	-	-	-
Curvularia (*Cochliobolus*)	4.76%	-	-	9.52%	-	6.67%	4.76%	5.55%	-
Fusarium	4.76%	-	-	-	-	6.67%	-	-	-
Mucor	-	5.55%	-	-	-	-	-	-	-
Ochrocladosporium	-	-	-	4.76%	-	-	-	-	-
Phomopsis (*Diaporthe*)	9.52%	5.55%	-	4.76%	27.78%	-	4.76%	16.67%	13.33%
Trametes	-	-	-	-	-	-	4.76%	-	6.67%
White mycelia sterilia	4.76%	-	6.67%	9.52%	5.55%	6.67%	-	11.11%	6.67%
Dark mycelia sterilia	-	-	-	9.52%	-	6.67%	-	5.55%	-
IF in each sample	23.80%	16.65%	6.67%	52.36%	33.33%	26.68%	23.80%	38.88%	26.67%
IF total in plant		15.07%			37.45%			29.78%	
Number of fungal taxa detected		6			9			7	

L leaf, *P* petiole, *S* stem

Molecular Identification

Based on molecular analysis, 46 selected endophytic fungi strains could be identified as *Alternaria, Cladosporium, Colletotrichum* (*Glomerella*), *Corynespora, Curvularia* (*Cochliobolus*), *Fusarium, Mucor, Ochrocladosporium, Phomopsis* (*Diaporthe*), and *Trametes*. Representatives of each fungal taxa were grown on PDA media as shown in Figure 2.

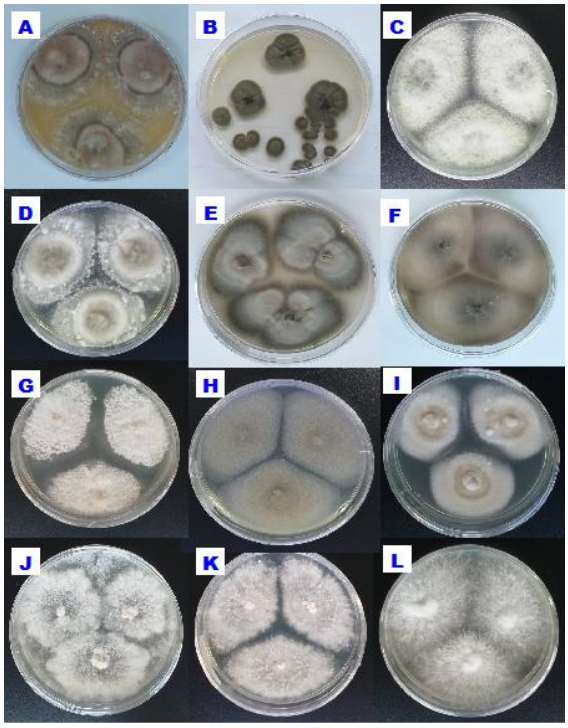

Figure 2. Macroscopic view of representatives fungal endophyte inhabiting *M. oleifera* from Lombok island, West Nusa Tenggara Province on PDA media, 7-10 d incubation at T 27 °C: (A) *Alternaria* ML2CL-1 (B) *Cladosporium* ML1BP-1 (C) *Colletotrichum* (*Glomerella*) ML3CL-1 (D) *Corynespora* ML2AL-2 (E) *Curvularia* (*Cochliobolus*) ML2CL-4 (F) *Curvularia* ML3BL-2 (G) *Fusarium* ML2BS-1 (H) *Mucor* ML1AP-1 (I) *Ochrocladosporium* ML2AL-5 (J) *Phomopsis* (*Diaporthe*) ML1CL-2 (K) *Phomopsis* ML2BP-2 and (L) *Trametes* ML3AL-1.

Phylogenetic Analysis

The phylogenetic analysis was conducted with the highest frequency of occurrence of endophytic fungi, namely *Curvularia* (*Cochliobolus*) and *Phomopsis* (*Diaporthe*). Based on the phylogenetic tree generated from the NJ analysis, the sequence of *Curvularia* sp. ML2CL-4 isolated from the leaf of *M. oleifera*

from Lombok island, West Nusa Tenggara, Indonesia nested in the same clade with *C. geniculata* strain CBS 187.50 (KJ909781), *C. soli* CBS 222.96 (KY905679), and *C. senegalensis* CBS 149.71 (HG779001) with 79% bootstrap value (Figure 3). The NJ tree of *Curvularia* species also showed that the sequence of *Curvularia* sp. ML3BL-2 isolated from the leaf of *M. oleifera* nested in the same clade with *C. verruculosa* strain CPC 28809 (MF490824), *C. verruculosa* strain CBS 149.63 (HF934909), *Cochliobolus verruculosus* strain ZTY98400 (HM053661), and *C. verruculosus* strain ZX991074 (HM053660) with 100% BS (Figure 3).

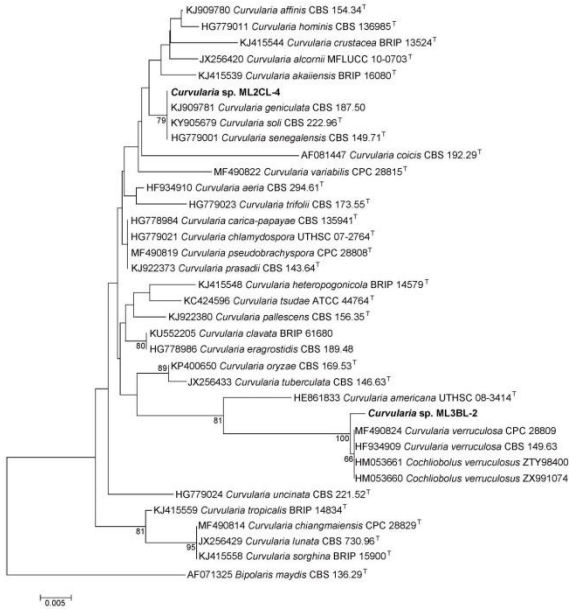

Figure 3. Neighbor-Joining (NJ) tree based on *Curvularia* spp. ITS sequences. Bootstrap values above 50% are recorded at the nodes (1000 replicates).

The NJ tree showed that the sequence of *Diaporthe* sp. ML2BP-2 isolated from petiole of *M. oleifera* from Lombok island, West Nusa Tenggara, Indonesia nested in the same clade with *D. perseae* strain ASHM300 (MK111099), *D. perseae* strain CBS 151.73 (KC343173), and *D. phoenicicola* strain SM30 (MN651492) with bootstrap value 99% (Figure 4). The sequence of *Diaporthe* sp. ML1CL-2 nested the same clade with *D. melonis* strain CBS 507.78 (KC343142) with 87% BS (Figure 4).

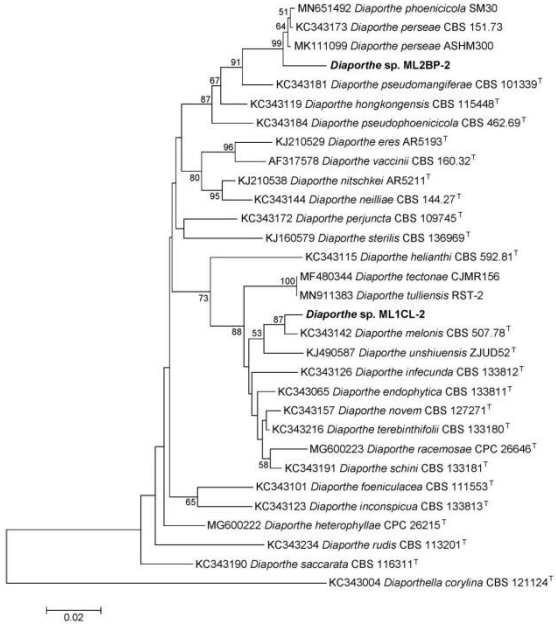

Figure 4. Neighbor-Joining (NJ) tree based on *Diaporthe* spp. ITS sequences. Bootstrap values above 50% are recorded at the nodes (1000 replicates).

Discussion

A total of 46 strains of endophytic fungi were isolated from 3 samples of *Moringa oleifera* collected from Lombok island, West Nusa Tenggara Province, consisting of 9 strains from *M. oleifera* sample I, 21 strains from *M. oleifera* sample II, and 16 strains from *M. oleifera* sample III. The percentage of colonization frequency (CF) results in Table 2 shows that endophytic fungi were mostly found in the leaves and stems segments of *M. oleifera* sample II with 52.37% and 100% colonization rate. The most abundant of endophytic fungi was obtained in *M. oleifera* sample II with a total colonization rate of 65.60%. A previous study conducted by Dhanalakshmi *et al*. (2013) reported that CF of endophytic fungi from *Moringa* leaves was 66.6%, while that in the stems sample was 25%.

Based on the isolation frequency (IF) from 3 different samples of *M. oleifera* (Table 3), a total of 10 fungal taxa were found. Among these, endophytic *Phomopsis* (*Diaporthe*) showed the highest IF at 27.78% in the petiole of sample II, and high IF at 16.67% in the petiole of sample III. Endophytic *Phomopsis* was also found in almost all of the three samples, except in stem samples of I and II.

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Besides endophytic *Phomopsis*, endophytic fungi *Curvularia* (*Cochliobolus*) were the most frequently obtained in the plant part of *M. oleifera* sample. In general, the highest total IF was found in sample II at 37.45%. Moreover, *M. oleifera* sample II also has the highest diversity of fungal endophyte with 9 different taxa were detected. The fungal endophyte community and composition were influenced by several factors such as the host plant species and the tissue types. Several fungal endophytic isolates indicate the significant host and tissue preferences (Li *et al.,* 2020).

The previous studies on endophytic fungi isolated from the tropical medicinal plants reported that some of the endophytic fungi which were frequently found are *Alternaria*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Diaporthe* (anamorph: *Phomopsis*), *Fusarium*, *Guignardia* (anamorph: *Phyllosticta*), *Lasiodiplodia, Pestalotiopsis, Schizophyllum*, and *Xylaria* (Praptiwi *et al*., 2016; Praptiwi *et al*., 2018; Ilyas *et al*., 2019; Praptiwi *et al*., 2020; Oktavia *et al*., 2020). Endophytic *Phomopsis* is the most abundant strain and often dominating the obtained strains (Praptiwi *et al*., 2018; Ilyas *et al.,* 2019). The population structure and distribution pattern of the obtained endophytic fungi are mostly influenced by environmental factors variation as well as the classification and genetic background of the host plant. The habitat of the host plant is the source of environmental factors that influences the structure and composition of microbial species that colonize roots, stems, branches, and leaves. Some of those environmental factors are temperature, humidity, geographic location, and the host plant vegetation (Jia *et al*., 2016).

A previous study conducted by Rajeswari *et al*. (2014) reported that endophytic fungi from *M. oleifera* leaves, stem, flowers, and calyx samples were predominantly by *Aspergillus* spp. However, in this study, endophytic fungi *Phomopsis* and *Curvularia* were the most frequently obtained in the plant parts of *M. oleifera* samples. *Curvularia* (*Cochliobolus*) was commonly found as endophytes, pathogens, and saprobes associated with various plants. In the previous study, *C. lunata* was isolated from *Sorghum bicolor* and it is thought to be one major cause of leaf spot disease in tropical planted sorghum (Hidayat & Ramadhani, 2019). *Curvularia soli* was only found on soil in Papua New Guinea (Marin-Felix *et al*., 2017). *Curvularia geniculata* was commonly found in tropical regions, *C. senegalensis* and *C. verruculosa* (*Cochliobolus verruculosus*) are distributed in subtropical and tropical regions (Farr & Rossman, 2020). The phylogenetic analysis based on the ITS rDNA sequence could not accurately identify the species of *Curvularia*. Therefore, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and the translation elongation factor 1-alpha (*tef1-α*) genes could be used to determine *Curvularia* species.

Phomopsis (*Diaporthe*) is also commonly found as endophytes, pathogens, and saprobes associated with diverse plants and is distributed worldwide (Gomes *et al*., 2013). *Diaporthe perseae* is isolated from leaves, fruit, and stems. *Diaporthe perseae* is distributed in Barbados, Malaysia, South Africa, and the Netherlands, while *Diaporthe phoenicicola* is found in India. *Diaporthe melonis* was isolated from *Cucumis melo* fruit in USA and soybean stem in Myanmar (Farr & Rossman, 2020). The ITS rDNA sequences could not be able to determine the identity of *Diaporthe* (*Phomopsis*) species. Therefore, β-tubulin (*tub2*) and translation elongation factor 1-α (*tef1-α*) genes are necessary to identify *Diaporthe* (*Phomopsis*) sequences accurately.

In conclusion, this study showed that leaves, petioles, and stems of *M. oleifera* have a wide diversity of endophytic fungi. Based on the percentage of colonization frequency (CF) results, the leaves and petioles have the highest amount of endophytic fungi compared to that of stems. Based on molecular identification, we identified 10 genera from 46 selected endophytic fungi isolates. *Curvularia* (*Cochliobolus*) and *Phomopsis* (*Diaporthe*) have the highest frequency of occurrence of endophytic fungi based on IF results.

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