

Isolation, Fruiting and Pathogenicity of *Marasmiellus palmivorus* (Sharples) Desjardin (comb. prov.) in Oil Palm Plantations in West Malaysia

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ABSTRACT

Malaysia's golden crop, oil palm (*Elaeis guineensis*), is susceptible to bunch rot disease caused by *Marasmiellus palmivorus* (Sharples) Desjardin (comb. prov.). Nonetheless, there is no published information on the morphology and pathogenicity of the species found in local oil palm plantations in Malaysia. Rhizomorphs and basidiocarps found on dead fronds or trunks were randomly sampled from the plantations located in the states of Perak and Selangor. Isolates were identified based on the morphology and molecular methods as *Marasmiellus palmivorus* and pure cultures subsequently produced similar fruit bodies (basidiocarps) by *in vitro* methods. Hyphal morphology was examined by light and scanning electron microscopy and found to be septate and produced clamp connections. White spore prints were obtained from each pileus. Naturally grown and induced basidiocarps were similar with diameter of pileus ranging from 1.0-2.8cm, slightly depressed at the centre, smooth, convex, with involute margin, orange-white fading to white and possessed a central, solid, cylindrical, tough, overall whitish stipes with length ranging from 0.8-2.6cm. The gills were adnate, distant and have a non-distinctive odour. Basidiospores were ellipsoid in shape and spores were found to be viable with percentage germination of 80-85%. Upon germination, they produced germ tubes ranging from 64.3 – 82.5 µm after 24 h incubation at ambient temperature ($27 \pm 2^\circ\text{C}$) on water agar. Pathogenicity test of six isolates of *Marasmiellus* sp. positively produced necrotic symptoms on wounded leaves of oil palm seedlings.

ARTICLE INFO

Article history:

Received: 25 May 2012

Accepted: 19 October 2012

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Keywords: Basidiocarps, basidiospores, bunch rot disease, *Marasmiellus palmivorus*, oil palm

INTRODUCTION

The oil palm industry is a major financial contributor to the economy of Malaysia. With cultivated areas of 5 million ha in 2011, RM80.4 billion revenues from the export of oil palm products were made (MPOB, 2012). Nonetheless, oil palm is susceptible to fungal diseases including bunch rot disease caused by *Marasmiellus* spp. This fungus belongs to the class of basidiomycetes in the order Marasmiaceae (Wilson & Desjardin, 2005). *Marasmiellus palmivorus* was previously known as *Marasmius palmivorus*. Hemmes and Desjardin (2002) found it more suitable to be grouped under the genus *Marasmiellus* based on its close morphology to other *Marasmiellus* species such as *Marasmiellus troyanus* and *Marasmiellus semiutus*. In addition, Wilson and Desjardin (2005) also revised the genus and tentatively accepted it as *Marasmiellus palmivorus* (Sharples) Desjardin comb. prov. until further phylogenetic analysis was done to support its accurate identification (D.E. Desjardin, personal communication, August 12, 2011).

Meanwhile, Sharples (1928) reported the first local outbreak of this disease as causing significant losses to oil palm. However, there has been no published information on the morphology and pathogenicity of this fungal species found in oil palm plantations in Malaysia. This paper reports the investigations made on the isolation, morphological characteristics of vegetative structures and fruit bodies, as well as fruiting behaviour of induced basidiocarps and pathogenicity of *Marasmiellus* isolates from oil palm plantations.

MATERIALS AND METHODS

Isolation of the Fungus

Random samplings of rhizomorphs and basidiocarps of fungus, associated with diseased oil palm fruits, fronds or trunks, were made in the states of Perak and Selangor (see Fig.1). For this purpose, four samples of the isolates from Selangor and two from Perak were obtained (Table 1). Fresh basidiocarps and rhizomorphs collected were washed in three changes of sterile distilled water. They were then

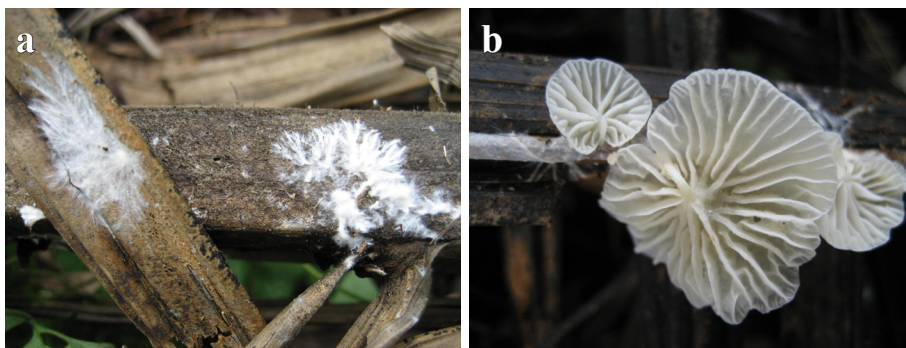


Fig.1: Random samplings of fungus associated with diseased oil palm. a. White rhizomorphs on dead oil palm fronds in Teluk Intan, Perak; b. Basidiocarps produced on dead oil palm trunks in Bangi, Selangor

TABLE 1
Marasmiellus isolates, collection information and GeneBank accession no.

Isolate	Location	Sample Type	GeneBank Accession no.
Bangi	Bangi, Selangor	Basidiocarp	JQ654222
Bangi1	Bangi, Selangor	Basidiocarp	JQ654223
Bangi3	Bangi, Selangor	Basidiocarp	JQ654224
UPM42	Serdang, Selangor	Basidiocarp	JQ654219
OP2	Teluk Intan, Perak	Basidiocarp	JQ654220
OP4	Teluk Intan, Perak	Rhizomorph	JQ654221

immersed in 20% Chlorox[®] solution for two minutes to eliminate contaminants and rinsed with three changes of sterile distilled water. Pieces of surface treated tissues (0.2cm x 0.2cm) were cut from the edge of the pileus and placed on Malt Extract Agar (MEA, Merck) plates and incubated at ambient temperature (27±2°C) for four days. The margins of vigorously growing colonies were subcultured and transferred onto fresh MEA slants and maintained at 4°C prior to further studies.

Morphology and Molecular Identification of Fruit Bodies and Vegetative Structures

Visual assessment of basidiocarps was carried out. Size, colour, shape and texture of pileus, stipe and gills were studied and identified using Sharples (1928), Turner (1981), Hemmes and Desjardin (2002) as source of references. Vegetative structures of isolates were described by examining hyphal structures on culture plates using light microscope and scanning electron microscope. Molecular identification of fungus was confirmed by extracting genomic DNA using the CTAB procedure and a large

subunit (LSU) region was amplified and sequenced using LR07/LR7 primers (B.S.A. Almaliky, personal communication, July 20, 2012) (see GeneBank Accession No. shown in Table 1).

In vitro Production of Basidiocarps

The *Marasmiellus* sp. isolates were first cultured for use as the source of spawn. Wheat grains of 0.5kg obtained from a wholesale market were soaked in water overnight, drained, transferred into a 1 L Scott bottle, tightly-plugged and autoclaved at 121°C, 1.05 kg/cm² for 20 mins. Upon cooling, 5mm mycelial discs of three-day old pure cultures were inoculated onto the grains aseptically for spawn-run.

Following the methods used to induce the fruit bodies of *Marasmiellus inoderma* (Sabet *et al.*, 1970) and *Marasmiellus scandens* (Ooi, 1987), empty fruit bunch (EFB) fibres from a local palm oil mill were used as substrates. The fibres were ground to pieces, autoclaved for one hour and cooled. Sawdust was similarly prepared and utilized as substrate for comparison. During the preparation, 2 kg of substrate was mixed thoroughly with 0.2 kg rice bran and 0.02 kg calcium carbonate (CaCO₃) (90% substrate:9% rice bran:0.9% CaCO₃). The mixture was then dispensed into polypropylene bags (15.2 cm x 33 cm) at 100g of substrate per bag, tightly capped with a stopper and autoclaved for 20 mins. After cooling, the substrate inoculated with a table spoon of fungal spawn and incubated in a glass chamber (92 cm x 46 cm x 30 cm) at ambient temperature

($27 \pm 2^\circ\text{C}$) for 30 days in total darkness. After that, the polypropylene bag was removed. Sterile distilled water was sprayed twice a day (at 0800h and 1700h) to maintain relative humidity of more than 85%. The substrate was exposed to 24 h of continual light (fluorescent white 20 W tubes), with normal alternating day and night conditions in the laboratory to study the effects of light on fruiting. There were four replications for each isolate, type of substrate and light exposure treatments. Meanwhile, fruiting was monitored by counting the number of basidiocarps produced over a period of four weeks. The experiment was a complete randomized treatment combination in a three factorial design. Spore print was prepared by placing overnight a freshly produced basidiocarp with the gills facing downward in a Petri dish containing a piece of black paper.

Viability of Basidiospores

Basidiospores collected from basidiocarps were dispensed into a vial containing 50

μL of sterile distilled water. One μL drop of spore suspension was placed on a glass slide layered with water agar, overlaid on a glass rod in a sterile Petri dish, sealed with a parafilm and incubated at $27 \pm 2^\circ\text{C}$. Basidiospore germination was assessed after 24 h of incubation by staining in lactophenol cotton blue. The percentage germination of basidiospores was recorded and germ tube growths were also measured.

Pathogenicity Tests

The tests were done on three-month old oil palm seedlings in a glasshouse at Universiti Putra Malaysia. First, a wound was created across each leaf by cutting a straight line using a needle. Basidiocarps that were borne on EFB substrate were supported and placed over each wounded leaf (see Fig.2). Transparent polypropylene bag was used to cover each seedling and inoculum. The tests were repeated for the basidiocarps produced by six isolates and replicated three times with three seedlings each. The relative humidity surrounding each seedling



Fig.2: Placement of *Marasmiellus* basidiocarps over wounded leaf of oil palm seedling in the pathogenicity tests

was monitored using a relative humidity meter at more than 85 % by spraying sterile distilled water twice at 0800 h and 1700 h daily. A period of two days was given to allow the natural dispersal of basidiospores and inoculation of the wounded leaf. The infectivity of the leaves was monitored and assessed over a period of four weeks. Fungal hyphae re-isolation from diseased leaves was obtained to confirm the presence of *Marasmiellus* sp. The percentage of disease incidence (I) was determined as follows:

$$I = \frac{\text{Number of plants infected per isolate}}{\text{Total number of plants per isolate}} \times 100\%$$

Statistical Analysis of Data

The data of the yield of basidiocarps, as well as the diameter of pileus and length of stipe, were subjected to the Analysis of Variance (ANOVA) test. The mean comparisons were done by using the Duncan Multiple Range of Test (DMRT). Data analysis of pathogenicity test was based on the transformed value obtained from

arc-sine transformation of disease incidence percentages.

RESULTS

Morphology and Molecular Identification of the Fruit Bodies and Vegetative Structures

Pure cultures of *Marasmiellus* sp. isolates produced dense, whitish, fan-shaped cottony mycelia with feathery edges on MEA plates when incubated at ambient temperature (27 ± 2 °C). The diameter of pilei ranged from 1.0-2.8cm and the length of the stipes were in the range of 0.8-2.6cm. Each pileus was slightly depressed at the centre, smooth, convex, with involute margin, orange-white fading to white. Stipe was central, solid, cylindrical, tough, and whitish, while lamellae were adnate, distant and have a non-distinctive odour (see Fig.1). Hyphae were septate and produced clamp connections under observations by light and scanning electron microscope (Fig.3).

All the isolates deposited in the GeneBank Accession (Table 1) showed

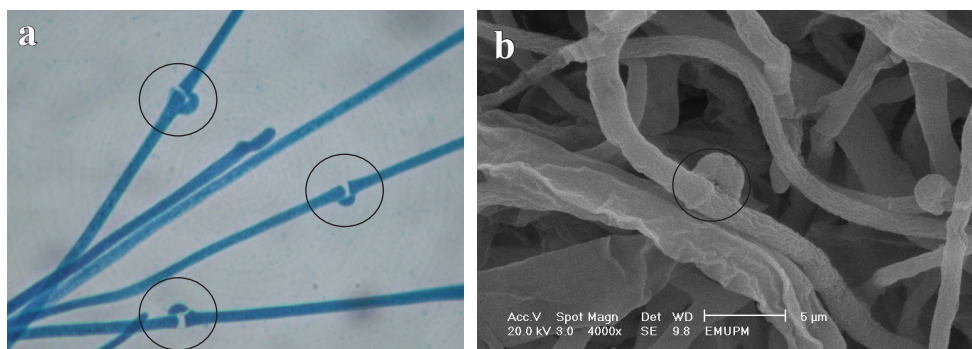


Fig.3: Hyphae of *Marasmiellus* sp. isolates; a. Appearance of septal walls and clamp connections (in circles) under the observation of light compound microscope; b. Presence of septate wall and clamp connection as shown (circle) by scanning electron microscope

sequences that were identical to each other and 99% similar to a *M. palmivorus* sequence deposited in the NCBI database (Accession No. AY639434) (Wilson & Desjardin, 2005).

In vitro Production of Basidiocarps

All six *Marasmiellus* sp. isolates from Perak and Selangor fruited on both EFB and sawdust substrate medium (see Fig.4). The statistical analysis showed that the yield of basidiocarps on EFB substrate was significantly higher than on the sawdust substrate under the conditions of complete darkness and normal alternate day night condition (Table 2). Nonetheless, fruiting did not occur on both the substrates with 24

h exposure to continuous white light. The diameter of the pileus on the EFB substrate was significantly larger than those found on the sawdust substrate (Table 3). However, there was also significant difference in stipe length of basidiocarps, where they were found to be longer on EFB compared to sawdust substrate (Table 4).

The morphology of *in vitro* produced basidiocarps of isolates on EFB and sawdust substrates was similar. Each pileus was slightly depressed at the centre, smooth, convex, with involute margin, orange-white fading to white, while stipe was central, solid, cylindrical, tough, and overall whitish. Lamellae were adnate, distant and have a non-distinctive odour (Fig.5). Spore prints were white, as shown in Fig.6.

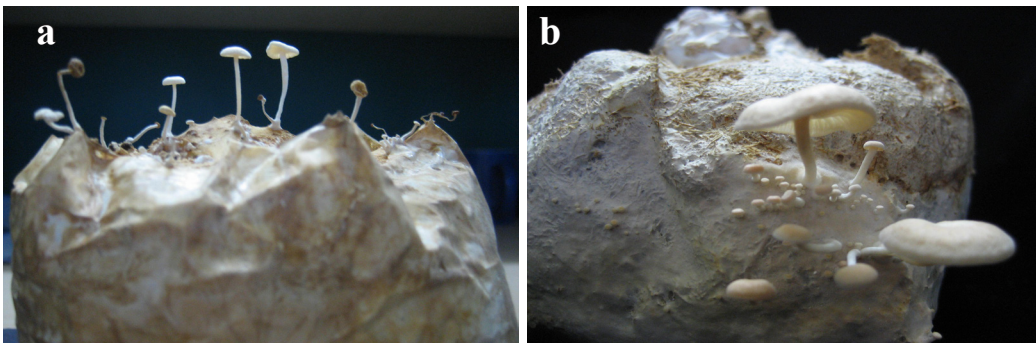


Fig.4: Fruiting of *Marasmiellus* sp. isolate; a. Smaller *Marasmiellus* basidiocarps on sawdust substrate; b. Larger sized *Marasmiellus* basidiocarps on oil palm empty fruit bunch substrate



Fig.5: A general appearance of basidiocarp (*Marasmiellus*) (bottom view) produced *in vitro* on the EFB substrate



Fig.6: Whitish spore prints (left) obtained from basidiocarp (*Marasmiellus*) (right)

TABLE 2
The effects of substrate on the number of basidiocarps produced by *Marasmiellus* isolates

Light exposure	Substrate	The mean number of basidiocarps produced according to light exposure of isolate per 100 g of substrate*					
		Bangi	Bangi1	Bangi3	OP2	OP4	UPM42
Complete darkness	EFB fibre	24a	24a	23a	20a	21a	22a
	Sawdust	9b	10b	8b	8b	9b	11b
Normal alternate day / night	EFB fibre	23a	23a	24a	22a	22a	21a
	Sawdust	10b	10b	9b	8b	8b	9b
24 h of continuous white light	EFB fibre	0c	0c	0c	0c	0c	0c
	Sawdust	0c	0c	0c	0c	0c	0c

*Mean of four replicates

Mean values with the same letters in the same column are not significantly different at 5% by DMRT

TABLE 3
The effects of substrate on the diameter of pileus (cm) of *Marasmiellus* isolates

Light exposure	Substrate	The mean diameter of pileus (cm) according to light exposure of isolate per 100 g of substrate*					
		Bangi	Bangi1	Bangi3	OP2	OP4	UPM42
Complete darkness	EFB fibre	2.60bc	2.56c	2.63bc	2.58c	2.66abc	2.59c
	Sawdust	0.74def	0.61g	0.58g	0.77de	0.60g	0.59g
Normal alternate day / night	EFB fibre	2.74ab	2.73ab	2.72ab	2.73ab	2.77a	2.73ab
	Sawdust	0.807d	0.67efg	0.65gf	0.67efg	0.675efg	0.66efg
24 h of continuous white light	EFB fibre	0h	0h	0h	0h	0h	0h
	Sawdust	0h	0h	0h	0h	0h	0h

*Mean of four replicates

Mean values with the same letters in the same column are not significantly different at 5% by DMRT

TABLE 4
The effects of substrate on the length of the stipe (cm) of *Marasmiellus* isolates

Light exposure	Substrate	Mean length of stipe (cm) according to light exposure of isolate per 100 g of substrate*					
		Bangi	Bangi1	Bangi3	OP2	OP4	UPM42
Complete darkness	EFB fibre	2.35ab	2.35ab	2.34ab	2.34ab	2.36ab	2.34ab
	Sawdust	1.32cd	1.23cb	1.20d	1.24cd	1.20d	1.23cd
Normal alternate day / night	EFB fibre	2.22b	2.48a	2.23b	2.22b	2.23b	2.23b
	Sawdust	1.45c	0.87c	1.12d	1.16d	1.17d	1.15d
24 h of continuous white light	EFB fibre	0f	0f	0f	0f	0f	0f
	Sawdust	0f	0f	0f	0f	0f	0f

*Mean of four replicates

Mean values with the same letters in the same column are not significantly different at 5% by DMRT

Viability of Basidiospores

The basidiospores observed under the light microscope were ellipsoid, with a size range of 6.2-8.7 μm (Fig.7a). They were viable with a percentage germination of 80-85% and germ tube growths recorded between 64.3 and 82.5 μm (see Fig.7b).

Pathogenicity Tests

The results of pathogenicity tests showed positive results with no disease incidence recorded in the uninoculated (control) oil palm seedlings and between 33 to 55% incidence of disease on all the seedlings inoculated by six *Marasmiellus* sp. isolates (Table 5). However, the uninoculated oil

palm seedling showed no development of disease as compared to the formation of necrotic lesions on the wounded leaf of inoculated seedlings (Fig.8)

TABLE 5
Disease incidence percentage recorded on control (uninoculated) oil palm seedlings and six isolates of *Marasmiellus* sp.

Test isolate	Disease incidence*(%)
Control (uninoculated)	0 ^b
Bangi (Bangi, Selangor)	55.5 ^a
Bangi1 (Bangi, Selangor)	44.4 ^a
Bangi3 (Bangi, Selangor)	44.4 ^a
OP2 (Teluk Intan, Perak)	33.3 ^a
OP4 (Teluk Intan, Perak)	33.3 ^a
UPM42 (Serdang, Selangor)	44.4 ^a

*Mean of three replications. Percentage values arc-sine transformed and analyzed for significance. Means followed by the same letter in the same column are not significantly different by DMRT at $P \leq 0.05$

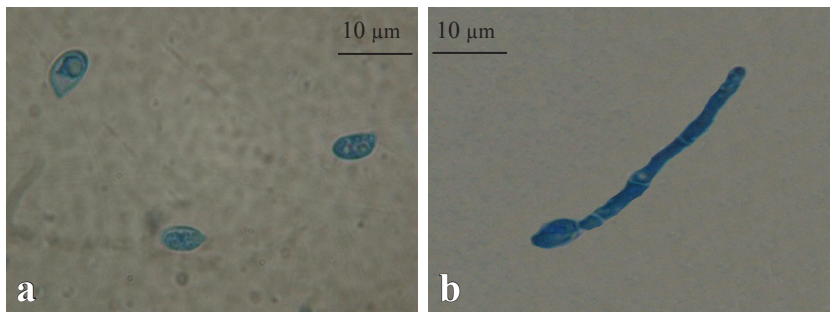


Fig.7: Basidiospores (*Marasmiellus*) observed under light microscope; a. Ellipsoid in shape; b. Germ tube growth on water agar at 24 h incubation at ambient temperature (27 ± 2 °C)

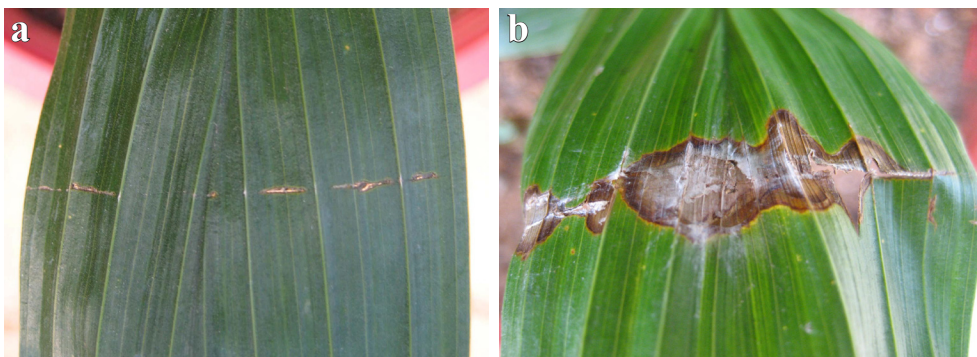


Fig.8: (a) Uninoculated oil palm leaf showing no development of disease; (b) Formation of necrotic lesions on wounded oil palm leaves at 13 days after inoculation with *Marasmiellus* sp. isolate

DISCUSSION

The morphology of rhizomorphs and basidiocarps of *Marasmiellus* sp. observed was found to be almost similar to those described by Sharples (1928), Turner (1981) and Hemmes and Desjardin (2002). Meanwhile, the presence of clamp connections in the hyphae of isolates used in this study were confirmed to have similar features with those described by Singer (1973) for *Marasmiellus* sp. Basidiocarps found in nature, while the *in vitro* produced in this study was shown to be generally smaller (1.0-2.8 cm) as compared to the sizes of natural basidiocarps (2.5-7.5 cm) described in the literature. This may be attributed to the amount of substrate available and the variability in the environment (Turner, 1981).

The fewer fruit bodies produced on the sawdust medium than the oil palm EFB substrate could be due to higher nitrogen content in the sawdust (1.64 %) compared to that in the EFB fibres (0.2-0.7 %) (Do, 1999; Mahlia *et al.*, 2000; Segura *et al.*, 2001). According to Hawker (1971), nitrogen is an important element involved during fruiting and a minimum amount of nitrogen will allow sporulation. On the contrary, excess amounts of nitrogen will promote active vegetative growth and inhibit fruiting. Carbon compounds have been known to play important roles in influencing fungal reproduction (Hawker, 1971). Although there could be higher carbon content in sawdust compared to EFB fibres (Do, 1999; Amal Nafissa *et al.*, 2008; Khor *et al.*, 2009), lower fruiting number in the former could

be due to lower carbon-to-nitrogen (C/N) ratio (50-75:1) in the EFB fibre compared to sawdust (Schuchardt *et al.*, 2002).

Carbon derived from cellulose, hemicellulose and lignin are the most abundantly utilized. In this study, the lower C/N ratio could be improved by addition of rice bran into the substrate medium used.

Meanwhile, some environmental factors such as light and nutrient availability were known to contribute to *in vitro* morphogenesis (Schwalb, 1978; Suzuki, 1979; Manachère, 1980). Light was found to be substantial in fungal fructification process and pileus differentiation (Plunkett, 1961; Kitamoko *et al.*, 1968, 1974; Perkins, 1969; Perkins & Gordon, 1969; Morimoto & Oda, 1973; Schwalb & Shanler, 1974; Raudaskoski & Yli-Mattila, 1985; Kaneko & Sagara, 2001). Since prolonged white light exposure has been shown to inhibit fruiting by *Marasmiellus*, the continuous illumination appeared to give a negative effect to the formation of primordia. Okwujiako (2001) found that light may inhibit the vegetative growth of some agarics but exposure of appropriate light duration is essential for the formation of basidiocarps. Dark condition has been shown to favour spawn run, while 12h of alternate light was found to be optimum for certain agarics during fruiting (Datta & Chakraborty, 2002).

The development of necrotic symptoms on leaves of oil palm seedlings after inoculation with basidiospores indicated that the *Marasmiellus* sp. isolates were pathogenic. However, the findings of this study showed variations in the incidence

of disease. Therefore, it is suggested that the quantity of inoculum of isolates should be quantified in order to obtain uniform infectivity in future work.

CONCLUSION

Bunch rot is an important disease of oil palm in Malaysia. This research has provided the first detailed documentation of the morphology *Marasmiellus palmivorus* (Sharples) Desjardin (comb. prov.) in oil palm plantations in Malaysia. In particular, the fruiting behaviour and pathogenicity of the local species on oil palm were elucidated.

ACKNOWLEDGEMENTS

The authors are grateful for the support given to this research through Fundamental Research Grant Scheme (Project no: 01-11-08-648FR) under the Ministry of Higher Education, Malaysia.

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