

ANATOMY OF SYMBIOTIC FUNGAL ENDOPHYTES IN *Psilotum nudum* (L.) P. Beauv

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ABSTRACT

A study was undertaken to identify the presence of fungal endophyte in fern plant, *Psilotum nudum*. Being the only living species of a once populated division, *P. nudum* is the most primitive vascular plant. Cross section of stipe and rhizoid parts was done to observe the presence of endophyte within parenchyma cells. Samples of *P. nudum* were collected at different sites in Bangi. Anatomical study showed *P. nudum* has percentage of infected cells highest in rhizoid parenchyma cortex (22.2%) and lowest in the apical area (3.1%). Eleven fungal endophyte isolates were successfully isolated and identified from *P. nudum* with *Aspergillus* being the major genus. Is *Aspergillus* a host-specific endophyte? The present study shows the compatibility of *P. nudum* parenchyma cells as a host to fungal endophyte.

Key words: Endophyte; anatomy; *Psilotum nudum*; parenchyma

INTRODUCTION

Plants interact with endophytes in mutualism and parasitism association, forming a complex relationship in time and space (Herrera *et al.*, 2002). Traditionally, ecological study of the relationship is examined separately by considering the pairing interaction between plants and pollinators such as, plants with mycorrhizal fungi and plants with parasites. However, this approach has changed completely in the last few years with the increasing number of studies that emphasize the importance of multiple interactions, including biota relations on the ground and give an understanding of the ecological and evolutionary processes in nature (Gehring & Bennett, 2009). However, the relationship that exists between plant parasites, mycorrhizal hosts are still less studied.

A plant relationship with mycorrhizal fungi is one of the most important relationships and is known as symbiosis. The type of interaction involved is mutualism which refers to the benefit gained by both parties (Elizabeth 1972). Plants are used as their host to live, they develop strategies in the chemical aspects that favour their existence. In exchange for carbohydrates from the plants, the endophytic fungi alter plant nutrient content and

enhance the production of secondary metabolites. Changes in chemical composition act to prevent the plant from being attack by insects or pests. (Jeffrey *et al.*, 2008). Endophytes usually inhabit above ground tissues like leaves, stem and bark which differentiates them from mycorrhizal symbionts. However, the differentiation is not strong because endophytes also can live in root tissue. Overall, endophytic fungi are ubiquitous and extremely diverse in host plants (Arnold *et al.*, 2003).

Psilotum nudum is an ornamental fern and also popular in traditional medicine (Figure 1 A & B). The benefits of *P. nudum* vary according to their parts. Oily spores of the species are given to infants to stop diarrhoea. The herb's juice showed antibacterial activity against *Micrococcus pyogenes* and *Pseudomonas aeruginosa* and also used as a purgative (Kumari *et al.*, 2011). Yamazaki *et al.* (2001) stated *P. nudum* contains flavonoids which have been associated with medicinal value on reduction of chronic disease (Ramos 2007). Flavonoids are a large family of plant secondary metabolites, principally recognized for their health-promoting properties in human diets (Hernandez *et al.*, 2009). The objectives of this study are to identify the parts of *P. nudum* consisting the highest percentage of fungal endophytes, to observe the modification of anatomical characteristic due to the

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presence of fungal endophyte and to identify the fungal endophyte present.

MATERIALS AND METHODS

Percentage of endophyte present

Fresh samples of the whole plant of *P. nudum* were taken from three different localities, Taman Paku Pakis UKM, (originally from Banting, Selangor), Rumah Tumbuhan UKM and Bandar Baru Bangi. A list of species studied is given in Table 1. Voucher specimens were kept at UKMB Herbarium. In this study, *P. nudum* were divided into five sections namely apex aerial (*aa*), below apex aerial (*baa*), middle aerial (*ma*), bottom aerial (*ba*) and rhizoid (*rh*) (Figure 1C). Specimens were fixed in acetic acid solution (1:3, acetic acid: 70% alcohol). Fixation, embedding and sectioning were performed following the methods described by Johansen (1940) and Sass (1958) while sectioning was done using a sliding microtome. Sections were stained in Safranin and Alcian Blue and then dehydrated in an alcohol series of 50%, 70%, 95%, and 100%. Slides were mounted in Euparal, then sections of the specimen were photographed using an Olympus Diaphlan microscope and images were processed using Analysis Docu Software (Johansen 1940; Saas 1958). Anatomical descriptions followed Ogura (1972) and the percentage of endophytic cells is counted by the modification formula of stomata counting by Metcalfe and Chalk (1957). The formula is based on the appearance of hypha endophyte which explained using this formula:

Percentage of endophytic cells:

$$\frac{\text{Cells with endophyte}}{\text{Cells with endophyte} + \text{Cells without endophytes}} \times 100$$

Isolation of fungi

Fungal isolation was done by using a surface sterilization method as proposed by Petrini *et al.* (1992) and sliced section (10mm x 10mm) was laid on Potato Dextrose Agar (PDA). Incubation was done for five days at 30°C. Lactophenol cotton blue wet mount preparation is used to demonstrate

fungi and fungal elements under microscope. Characterisation of endophytic fungi was carried out by macroscopic and microscopic observations. Parameters for macroscopic observation include colony color and diameter, pigmentation, presence of pigment diffusion in agar, mycelium texture and colony edge. Microscopic observation at 400x magnification was done to observe the structure and presence of hyphae, septa end, asexual spores and sexual spores.

RESULTS AND DISCUSSION

Percentage cells with fungal endophyte presence

The sections were calculated according to the frequent appearance of hyphae (h) and hyphal coil (hc) in parenchyma tissue. Based on result, it shows that frequency of endophytes presence is highest in the *rh* part. High density fungal endophytes colonies were observed in the *baa*, *ma*, *ba* and *rh* parts. *Rh* part showed to have the highest percentage of endophytes colonization by 22.22% compared to others (Table 2). The abundance of endophytes at the *rh* correlates with the position of this structure which is closest to the ground. This high colonization of endophytic fungi can be considered of the spread and dominance of fungal endophytes through soil nutrient uptake (Rodriguez *et al.*, 2008). Therefore, it is possible that a sign of possibility that fungal endophytes found in *P. nudum* are strongly derived from the surrounding habitat or area. The presence of fungal endophytes has been previously reported by Peterson *et al.* (1981) on the gametophyte of *P. nudum* that found abundant fungal endophyte in rhizoid and corticol parenchyma in most cells. Findings from this study agreed with Vega *et al.* (2010) where the highest frequency of colonization by endophytes is at the rhizoid.

Results have shown that parenchyma cells containing endophytes normally have thicker cell walls. This is due to the structure of the haustorium hyphae that penetrate through the pores of the wall in parenchyma cells, in order to absorb water and nutrients from the host (Bhandari & Mukherji 1993). Admission hyphae and mycelium structure accordingly will improve parenchyma cells wall

Table 1. List of species studied

No.	Species	Locality	Part used	Date of collection & collector
1	<i>P. nudum</i> AF1	Taman Paku Pakis UKM (Bangi, Selangor)	Aerial & rhizoid	05.05.11 Ruzi Rahman Affina Eliya
2	<i>P. nudum</i> AF2	Rumah Tumbuhan, UKM	Aerial & rhizoid	05.05.11 Ruzi Rahman Affina Eliya
3	<i>P. nudum</i> AF3	Bandar Baru Bangi area	Aerial & rhizoid	05.05.11 Ruzi Rahman Affina Eliya

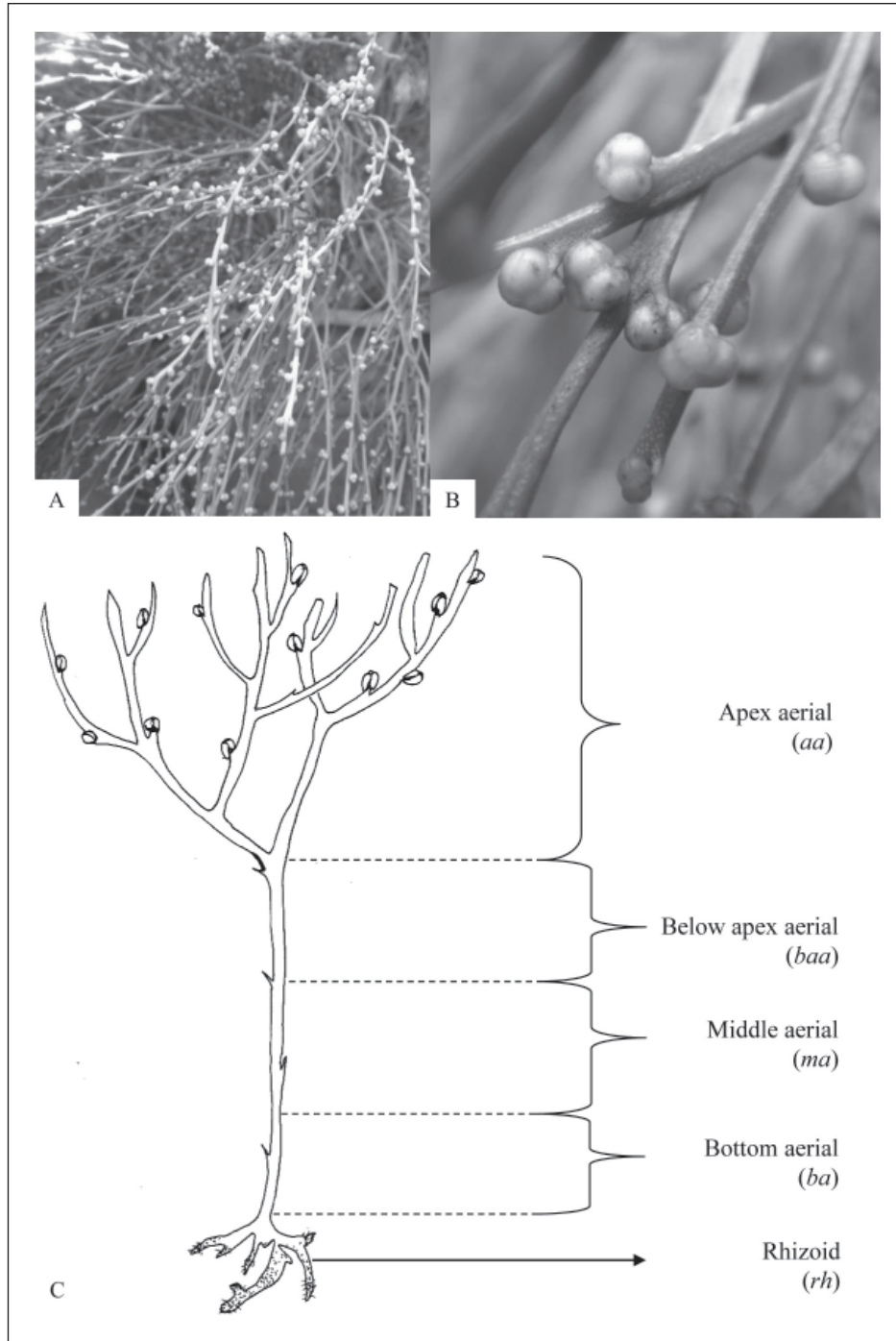


Fig. 1. *Psilotum nudum* (L.) P. Beauv. (A) Aerial Morphology, (B) Synangium. Source: Taman Paku Pakis UKM Bangi (C) Diagram of plant parts.

Table 2. Percentage density of endophyte in different part of *Psilotum nudum*

No.	Parts	Cell with endophyte	Cell without endophyte	Percentage (%)
1	Apex aerial (aa)	10	311	3.1
2	Below apex aerial (baa)	22	549	3.8
3	Middle aerial (ma)	54	623	7.9
4	Bottom aerial (ba)	60	412	12.7
5	Rhizoid (rh)	94	329	22.2

Table 3. Characterisation of endophytic species in *Psilotum nudum*

Parts	n	Macroscopic Characteristics			Microscopic Characteristics			Endophyte identification
		Colony colour	Colour beside colony	Micelium texture	Hypha structure	Micelium structure	Spore structure	
Apex aerial (aa)	1	LY	Nill	Pd	None	None	None	None
Below apex aerial (baa)	1	W	WDBS	Pd	As	As	Conidiophore aerial round, conidia forming chain structure	<i>Aspergillus niger</i>
	2	DB	DB	Ct	As	As	Conidiophore aerial round compact column.	<i>Aspergillus terreus</i>
	3	W	WDBS	Pd	As	As	Conidiophore aerial round, conidia forming chain structure	<i>Aspergillus niger</i>
	4	W	W	Ct	S	S	Conidiophore geniculate, conidia fusoid-shaped and consists of 5 cells.	<i>Bipolaris</i> sp.
	5	W	W	Ct	S	S	Arthroconidia unicellular and oval-shaped has large diameter.	<i>Coccidioides immitis</i>
Middle aerial (ma)	1	W	W	Ct	S	S	Ovoid shaped arthroconidia.	<i>Paracoccidioides brasiliensis</i>
	2	W	W	Ct	S	S	Ovoid shaped arthroconidia.	<i>Paracoccidioides brasiliensis</i>
	3	W	W	Ct	S	S	Conidiophore simple structured, single-celled conidia and ovoid shaped.	<i>Verticillium</i> sp.
	4	W	W	Ct	S	S	Conidiophore simple structured, single-celled conidia and ovoid shaped.	<i>Verticillium</i> sp.
Bottom aerial (ba)	1	W	W	Ct	As	As	Sterile mycelium structure.	Deuteromycota
	2	W	W	Ct	As	As	Sterile mycelium structure.	Deuteromycota
	3	W	Gy	Ct	As	As	Conidiophore simple structured, single-celled conidia and ovoid shaped.	<i>Verticillium</i> sp.
	4	Gn	W	Ct	As	As	Conidia are round and chain.	<i>Aspergillus flavus</i>
Rhizoid (rh)	1	W	W	Ct	S	S	Conidiophore branched, and unicellular conidia obovoid shaped.	<i>Scedosporium apiospermum</i>
	2	W	DGy	Ct	As	As	Sterile mycelium structure	Deuteromycota

Amendment: LY=Light yellow, W=White, WDBS=White and dark brown spot, DB=Dark Brown, Gn=Green, Gy=Grey, Pd=Powdery, Ct=Cottony, As=Aseptate, S=Septate.

thickness. It has also been reported by Vega *et al.* (2007) which stated that endophytic parenchyma cells were observed in the thickened parenchyma cells wall. Observations on thin-walled parenchyma cells also did not show the presence of any structures and no endophytic hyphae were observed to dominate the area.

Parenchyma cells that contain endophytes were observed to have dark cells. These factors caused by fungal endophytes cells that absorb more Alcian Blue staining solution compared to those cells without endophytes. Based on the study of Vega *et al.* (2007), the cells cross section of *Cytinus salviifolius* (angiosperm) in the presence of endophytes, visibly coloured dark blue more than other cells in *C. salviifolius* that not contain endophytic hyphae structures. Observations to determine the presence of endophytes in

parenchyma cells can simply be made by comparing the cell color, cell sizes and also confirmation of attendance through observation under a light microscope with high magnification.

Anatomical features are characterised by cells, tissues or internal structure of specific plants species. In the species studied, anatomical features are the presence of wide parenchyma cells with intercellular spaces. These anatomical characteristics are considered to have some sort of influence and impact on the presence of fungal endophytes (Figure 2). It has also been discovered by more than 100 years of research that the habitat where the plant species grow and breed also influenced the presence of endophytic fungi in the species (Petrini 1986). However further study from various localities must be done to prove this.

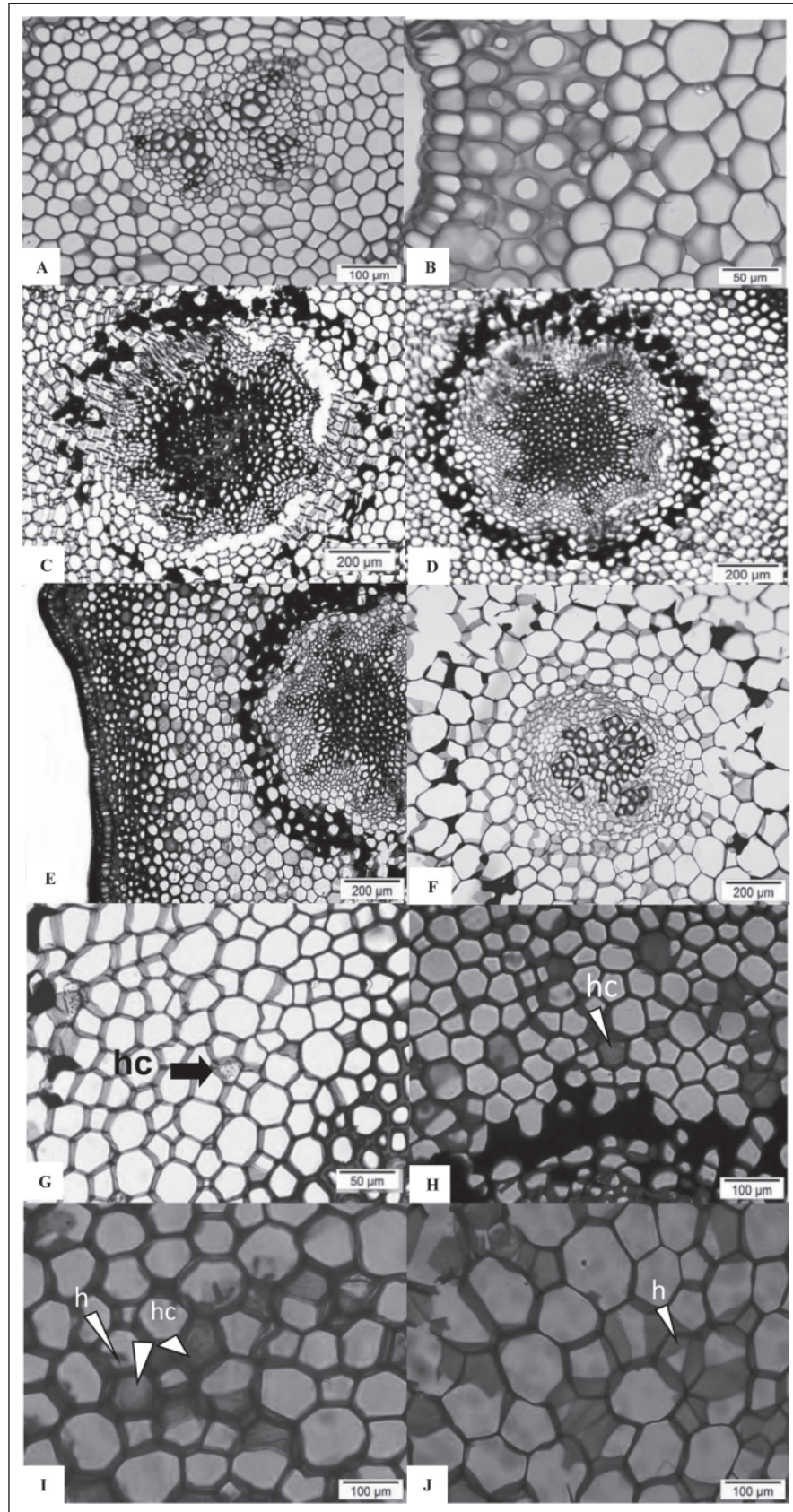


Fig. 2. Anatomical characteristics of *Psilotum nudum* parts with the presence of endophytes. (A, B) Cross section of apex aerial without endophytes. (C, D, E, F) Cross section of stele surrounded with endodermis cell layer. (G, H) Cross section of *baa* and *ma* with hyphae coils. (I) Cross section of *ba* with hyphae and hyphae coils. (J) Cross section of *rh* with hyphae. hc, hyphae coil; h, hyphae.

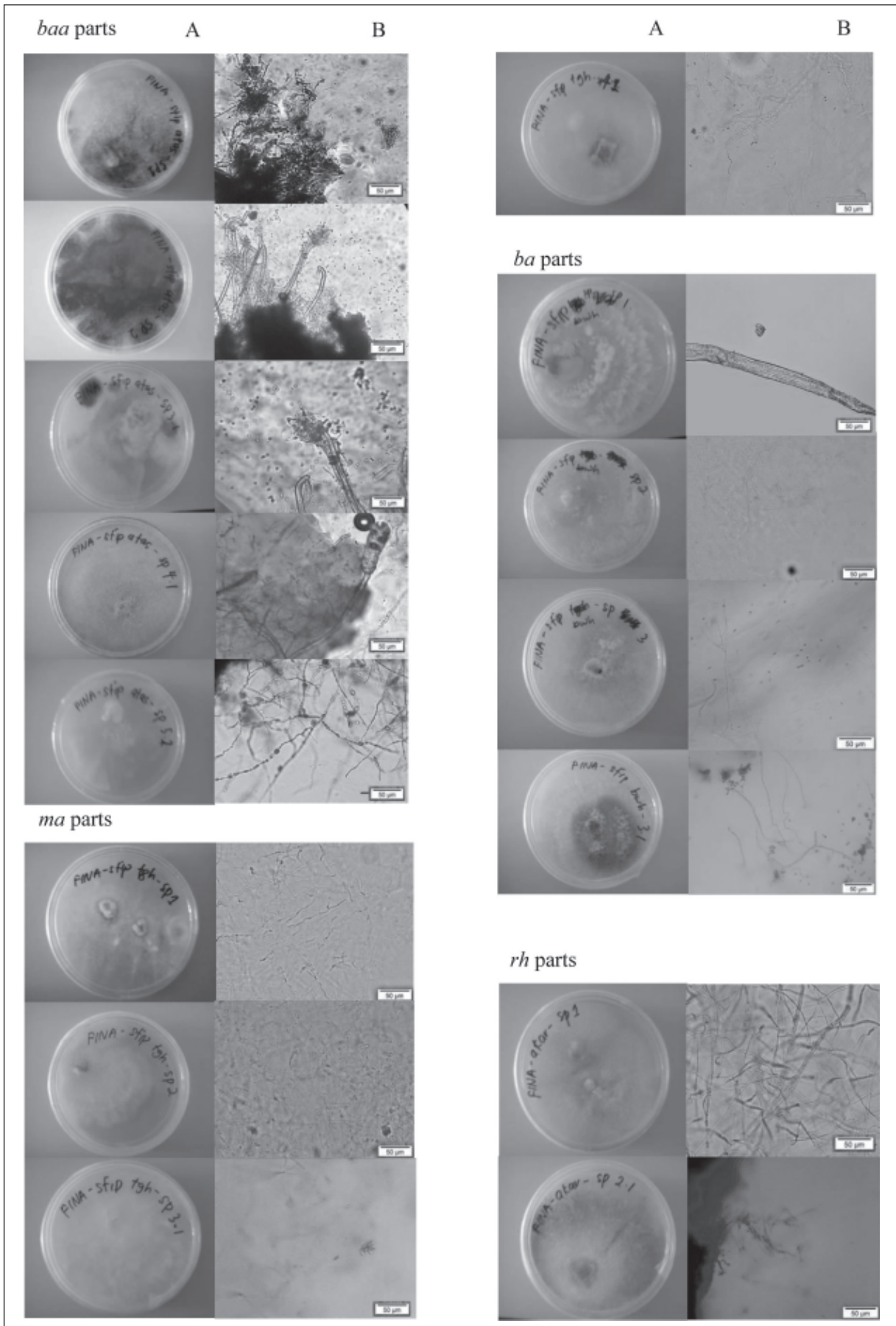


Fig. 3. Fungal isolates (A) Macroscopic observation on PDA agar. (B) Microscopic observation with 400x magnification.

Fungal Isolates Characteristics

Fungal endophyte isolates were successfully identified using macroscopic and microscopic observations (Table 2, Figure 3). The identifications were based on reference book of *The Identification of Fungi and Identifying Filamentous Fungi*. All fungal isolates have their own unique colony colors which can be classified into different group. Eleven colonies were successfully identified and categorized based on parts of the species where *Aspergillus niger*, *Aspergillus terreus*, *Coccidioides immitis* and *Bipolaris* sp. were present on *baa* parts, *Paracoccidioides brasiliensis* and *Verticillium* sp. present on *ma* part, *Aspergillus flavus*, *Verticillium* sp. and Deuteromycota class was found on *ba*, *Scedosporium apiospermum* and isolates from Deuteromycota class was observed in *rh* parts.

CONCLUSIONS

This study proved that fungal endophyte colonization assist the plant cells adaptation in the protection against biotic or abiotic stress. These lead to beneficial properties of fungal endophytes either in the increasing of plant development or adapting plant to unsuitable growth conditions. Hence, symbiotic support from fungal endophyte gave plant a long life time span.

ACKNOWLEDGEMENTS

We wish to thank the staff of Taman Paku Pakis UKM for contributing samples for the study. Deepest thanks are also to Mohamad Ruzi Abdul Rahman for guidance in the field. This research was supported by Grant DPP-2015-144 and DPP-2015-077.

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