

ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI OF *Pandanus sp.* AND *Alpinia sp.* FROM RESERVE FOREST UiTM NEGERI SEMBILAN

Nor' Aishah binti Hasan* and Maizura binti Zaini

¹*School of Biological Sciences, Faculty of Applied Sciences
University of Technology MARA, Negeri Sembilan Branch, Kuala Pilah Campus, 72000 Kuala Pilah, Negeri Sembilan,
Malaysia*

*Corresponding author: aishahnh@uitm.edu.my

Abstract

Endophytes are an endosymbiont that colonize in plant without causing apparent disease. Medicinal plants have been proven as a host to endophytes fungi thus providing a rich reservoir of bioactive compounds. However, there is lack information available about medicinal plants associated endophytes. Therefore, in the present study, the presence of endophytic fungi inhabiting in leaves of *Pandanus sp.* and *Alpinia sp.* were isolated. Healthy and symptomless leaves of *Pandanus sp.* and *Alpinia sp.* were surface sterilized and used for the isolation of endophytes. Variation in the colonization frequency of endophytic fungi was observed in leaves of both species. Relatively, leaves of *Pandanus sp.* were found to have greater colonization frequency (16.7%) as compared to leaves of *Alpinia sp.* (8.3%). A total of 3 fungal isolates were obtained and identified based on the morphology of fungal cultures and spores. Among them one unidentified isolate (P2) was group to Zygomycota phylum. Other two isolates were identified as species belong to *Lentinus sp.* (A1) and *Colleotrichum sp.* (P1). Finding in this study suggest the possible exploration of many such bioactive compounds from this potential fungus.

Keywords: *Alpinia sp.*, *Colleotrichum sp.* endophytic fungi, *Lentinus sp.*, *Pandanus sp.*

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Introduction

Endophytes are minute organisms (bacteria, fungi and actinomycetes) that lives within a plant and causes no harm to the host plant (Rajeswari et al. 2017). Among them, fungi are the most frequently recovered endophytes isolated from wild or cultivated crops either the monocot or dicots. Fungal endophytes form a unique specific symbiotic relationship by protect their hosts from contagious agents and withstand at adverse conditions by discharging active metabolites (Chandra, 2012). Plants infected with fungal endophytes are often healthier than endophyte free ones (Pańka et al., 2013).

Theoretically, to date, almost all types of vascular plants contain various and numerous endophytes in their tissues. Diversity of endophytes fungal are influenced by geographical location, climatic patterns, physiology and specificity of colonized tissue (Sivaramakrishnan et al. 2015). It is remarked as an important source of bioactive natural products due to occupying millions of unique biological niches growing in different types of environment (Maarof et al. 2012). Previous study have been reported the isolation of endophytes fungal from many different plants including trees (pine and yew), fodders (alfalfa, sorghum and clover), vegetables (carrot, radish, tomatoes, sweet potatoes, lettuce, and soybean), fruits (banana, pineapple, and citrus), cereal grains (maize, rice, and wheat), and other crops (sugarcane, marigold, and coffee) (Vijaya et al. 2013). However, in the past decade, there has been renewed attention on endophytes fungal in medicinal plants. It is gaining global attention owing to the fact that the herbal drugs are cheap, ubiquitous and safer. Among of medicinal plant which has not yet explored for endophytic fungal communities are *Pandanus sp.* and *Alpinia sp.*

Pandanus sp. belongs to monocotyledonous plants. It is widespread in tropical and sub-tropical areas such as Malaysia, Pacific islands and also Australia (Laluces et al., 2015). It is often used as herbs and as medicines while several species are cultivated as ornamentals. The green leaf fibre of *Pandanus* is sometimes used for weaving into mats and baskets, as well as for house thatch. Their associated endophytic fungi were first studied by McKenzie et al. (2002), with further research conducted by Thongkantha et al. (2008), Ariffin (2013), Bungihan et al. (2013) and Tibpromma et al. (2018). *Alpinia sp.* is a genus of flowering plants in the ginger family, Zingiberaceae. Species are native to Southeast Asia and Middle East including Malaysia (Kaushik et al., 2011). *Alpinia sp.* or any other plants species from Zingiberaceae family can be characterized by its aromatic properties can be used as medicine, ornamental, food and cosmetic industries (Azeman, 2009).

Medicinal plants are reported to harbor endophytes which in turn protect the host from infectious agents and survive the plants from adverse environment (Jariwala & Desai, 2018). The diversity of endophytic fungi, their population dynamic, their role in plant growth, plant health, their distribution in the plant, the secondary metabolites they secrete and their potency to produce novel compound within plants have formed an alarming aspect in this present study. Furthermore, in Malaysia, there is no study reported the diversity of endophyte fungi in *Pandanus sp.* and *Alpinia sp.* Therefore, efforts have been made to isolate the endophytic fungi inhabiting in leaves of *Pandanus sp.* and *Alpinia sp.*

Methods

Collection of plants

The symptomless and apparently healthy leaves (disease-free parts) of *Pandanus sp.* and *Alpinia sp.* were randomly collected from UiTM Negeri Sembilan Forest Reserve. For each plant, twelve leaves were collected, totaling a sample of twenty-four leaves which were immediately subjected to endophytic fungi isolates. The plant materials were collected in a sterile bag and was processed in the laboratory immediately to minimize the chances of contamination.

Isolation of endophytic fungi

The plant materials were taken and rinsed in running tap water to remove the dust and debris. Segments of approximately 0.5 cm were cut in sterile lancet blades and surface sterilized by agitating in 70% ethanol (1 min), followed by treatment with 4% NaOCl (90 s) and then rinsed in sterile distilled water (10 s) recommended by Sandhu et al. (2014). The plant samples were rinsed in distilled water for three times and were allowed to dry on filter paper. The surface-sterilized segments were cut into 5mm x 5mm segment using a sterile scalpel and 5 replicates were performed for each medium were then placed onto Potato dextrose agar (PDA) supplemented with chloramphenicol 150 mg/l. The Petri dishes were sealed using parafilm and incubated at 28°C. The fungi that grown out from the tissues was isolated and stocked. PDA slants were used to preserve cultures at 4°C for further screening (Satpute & Vanmare, 2017).

Identification of endophytic isolates

Morphological features including colony morphology, pigmentation, growth pattern, spore structures, and other hyphal characteristics were used to identify the endophytic fungal isolates with aid of standard mycological manuals (Ellis and Ellias, 1971; Bacon and White, 2000). Reproductive spores were examined under microscope. Minimal media were used to grown cultures which failed to produce spores and incubated for several weeks to months.

Colonization frequency analysis (CF)

Frequency of fungal endophytes harbored in plant species were calculated to determine the endophytes richness by the number of segments colonized by endophyte species divided by a total number of segments examined $\times 100$ (Divya et al. 2017).

Colonization frequency (CF%):

$$CF = \frac{\text{No of individual fungi recorded}}{\text{Total number of segments examined}} \times 100$$

Total no of segments screened

Result and Discussion

This study was carried to isolate and identification of endophytic fungi from Reserve Forest of Universiti Teknologi MARA, Cawangan Negeri Sembilan. The plant tissues specially leaves and stems are excellent reservoirs for endophytic fungi. About twenty-four leaves sample (twelve samples of each species respectively) of the *Pandanus sp.* and *Alpinia sp.* were screened for the isolation of the endophytic fungi (Figure 1). A total of three endophytic fungi was isolated and identified from both medicinal plants. The leaf samples from *Pandanus sp.* showed a maximum repository for endophytic fungi than the *Alpinia sp.* Among the three endophytic fungi, one unidentified isolate (P2) was group to Zygomycota phylum. Other two isolates were identified as species belong to *Lentinus sp.* (A1) and *Colleotrichum sp.* (P1). These results were similar with Meifei et al. (2013), who isolated *Colleotrichum sp.* in *Pandanus amaryllifolius*. However, Orlandelli et al. (2012), reported the isolate belongs to *Fusarium*, *Xylaria*, *Pestalotiopsis*, *Aspergillus*, *Nigrospora*, *Stachybotrys*, *Rhizoctonia*, and *Macrophomina* in 500 sample of leaves of *Pandanus sp.* Li et al. (2011) also found a various endophytic fungus in *Alpinia officinarum* including *Pestalotiopsis*, *Sebacina*, *Penicillium*, *Marasmius*, *Fusarium*, *Exserohilum*, *Mycoleptodiscus*, *Colleotrichum*, *Meyerozyma*, and *Scopulariopsis*.



Figure 1. Plants selected for the present study. (A) *Pandanus sp.*, (B) *Alpinia sp.*

Tables 1 showed the CF value of endophytic fungi. *Pandanus sp.* showed the highest percentages of endophytes fungi isolated compared to *Alpinia sp.* However, both species demonstrated lower endophyte richness with only fewer number of isolated found. Negative result of the study indicates that the Reserve Forests UiTM Cawangan Negeri Sembilan is an undistributed environment. This confirms the assumption that the more stress a plant receives in an environment the more susceptible it is to endophytes (Carbungco et al. 2017). Moreover, environmental factors such as temperature, rainfall, altitude and atmospheric humidity and their effect on host plant made the variations in occurrence of endophytic fungi and their colonization frequency (Selim et al. 2012). According to Rajagopal and Suryanarayanan (2010), low rate of colonization of endophytic fungi as observed in this study may be attributed due to the secretion of the certain antifungal and antibacterial components.

Table 1: Endophytic fungi isolated from *Pandanus sp.* and *Alpinia sp.*

Host	No. of samples	Number of fungi isolated	CF	Identified as	
				Phylum	Genus
<i>Pandanus sp.</i>	12	P1	8.3%	Zygomycota	-
		P2	8.3%	Ascomycota	<i>Colleotrichum sp.</i>
		A1	8.3%	Basidiomycota	<i>Lentinus sp.</i>

CF: Colonization frequency

Table 2 showed that, macroscopic examination indicated that among the three endophytic fungi isolates, P1 showed slowest growth rate as colonies takes 14 days to fully occupy the petri dish

whereas P2 showed rapid growth rate as colonies reaching 3–5 cm diameter on PDA after 3 days. A1 isolate showed a moderate growth rate with 5 days colonization period. Figure 2 shows the colony and spore appearance of some of the identified fungal endophyte in the leaves of *Pandanus sp.* and *Alpinia sp.* Sample of P1 from *Pandanus sp.* appeared greyish-green color in the center with cottony aerial mycelium and white concentric rings on PDA. P2 demonstrated white, flat colony with cottony texture on PDA while A1 showed white, velvety texture and slightly pigmented aerial mycelium on PDA the petri dish.

Table 2: Cultural characteristics of the 3 endophytic on PDA.

Isolate	Host	Size of colony		Shape	Color	Mycelium
		3 days	14 days			
P1	<i>Pandanus sp.</i>	A	Circular	greyish-green	Aerial
P2	<i>Pandanus sp.</i>	3–5 cm	>A	Circular	white	Flat
A1	<i>Alpinia sp.</i>	A	Circular	white	Aerial

Notes: >A completely covering plate, <B less than 1 cm

The conidia of sample P1 from *Pandanus sp.* demonstrated cylindrical with bluntly rounded ends shaped and slightly constricted at the middle. The ascocarps or the fruiting body has shape like a spade. The hyphae when observed under microscope appears septate and branched with many vacuoles can be seen clearly as shown in Figure 1. Sample P2 from *Pandanus sp.* showed the fruiting body, or the sporangium appeared round under the microscope. The sporangium is supported by the long stalk, sporangiophore. The apophysis that supported the sporangium was present. However, the conidia or the spores were not observed due to the age of the endophytes plate was more than 7 days. Sample A1 spores appeared to be subglobulose, small and may grow in dense clusters along the hyphae. The hyphae appeared aseptate and have branched filament.

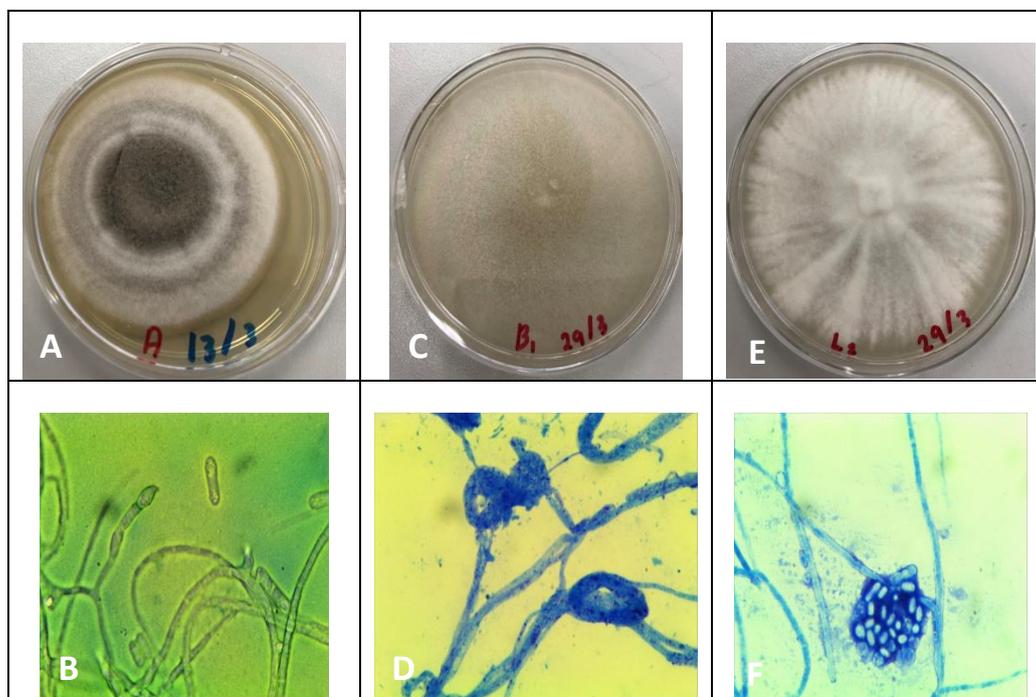


Figure 2. The morphology (colony appearance, conidia, and hypha) of some endophytic fungi isolated from *Pandanus sp.* and *Alpinia sp.* (A -B) sample P1 from *Pandanus sp.*, (C-D) sample P2 from *Pandanus sp.*, (D-E) sample

A1 from *Alpinia sp.*

Findings in this study demonstrate the presence of endophytic fungi from both medicinal plants with limitation to identify the potential endophytic fungi at the species level. Based on the classical mycology approach, the use of morphological features was problematic for phylogenetic systematics of hypogeous ascomycetes due to small set of morphological characteristics and homoplasy (Barseghyan and Wasser, 2010). Therefore, microscopic characteristics and confirmation by using ITS-rDNA sequence is urgently needed for unclear species identification. Nowadays, the use of next generation sequencing (NGS) is becoming popular and another option to identify fungi that cannot be cultured *in vitro*. In summary, result in this study indicate the presence of endophytic fungi in their *Alpinia sp* and *Pandanus sp.* host. Further plant bioactivity analysis is required to identify the bioactive compounds within the plants tested with bioactivity potential.

Conclusion

In the present study, three endophytic fungi were successfully isolated from *Pandanus sp.* and *Alpinia sp.* The sample P1 was identified as *Colletotrichum sp.*, sample P2 was classified under Phylum Zygomycota and sample A1 was identified as *Lentinus sp.* Further investigation will focus to exploit their potentiality to develop as an antimicrobial/ chemotherapeutic agent.

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