Malaysian Journal of Microbiology, Vol 19(1) 2023, pp. 55-62
DOI: http://dx.doi.org/10.21161/mjm.221535

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Isolation and morphological identification of endophytic fungi from leaves and stems of *Aquilaria malaccensis* Lamk. originating from Java Island, Indonesia

Abdul Salim[1] and Tri Rini Nuringtyas[2,3]*

[1]Graduate Study Program, Faculty of Biology, Universitas Gadjah Mada, Jl Teknika Selatan, Sekip Utara, Yogyakarta, 55281, Indonesia.
[2]Tropical Biology Department, Faculty of Biology, Jl Teknika Selatan, Sekip Utara, Yogyakarta, 55281, Indonesia.
[3]Research Center for Biotechnology, Universitas Gadjah Mada Jl. Teknika Utara, Kocoran, Yogyakarta, 55281, Indonesia.
Email: tririni@ugm.ac.id

Received 29 May 2022; Received in revised form 15 November 2022; Accepted 2 January 2023

ABSTRACT

Aims: Endophytic fungi are the remarkable category of host-associated fungal community that invades the intercellular regions of host tissues, benefiting their host while obtaining an advantage. Fungal endophytes have lately attracted prominence as a source of active secondary metabolites. This investigation aimed to identify fungal endophytes that reside inside the leaves and stems of *Aquilaria malaccensis*.
Methodology and results: Healthy *A. malaccensis* stems and leaves samples were collected. Clean leaves and stems were cut to a size of 1 cm, followed by sterilization using 75% ethanol for 1 min, 3% sodium hypochlorite solution for 1 min, and finally, rinsing with sterile water 3 times for 1 min and drying with sterile paper. The sterile samples were put onto Potato Dextrose Agar (PDA) media containing chloramphenicol for 7-14 days until the mycelium grew for morphological identification under a light microscope. Five endophytic fungi were recovered from leaves, while nine endophytic fungi were obtained from stems. Using morphological approaches, nine of the endophytes had observed to produce conidia fungi, whereas the others did not. *Neopestalotiopsis* sp., *Aspergillus* sp., *Arthrinium* sp., *Curvularia* sp., *Podospora* sp., *Mucor* sp. and *Verticillium* sp. were identified as nine of the fourteen endophytes.
Conclusion, significance and impact of study: The number of endophytic fungi discovered in different organs varies. Not all endophytic fungi that grow can create sexual phages. Six genera of endophytic fungi were identified.

Keywords: Aquilaria malaccensis, endophytic fungi, agarwood, fungal identification

INTRODUCTION

Plants are residents of various microorganisms. The microorganisms that colonize the plants can be classified into epiphytes which are available on a superficial level, endophytes that are situated inside the plant tissues; and rhizospheres which live in the soil near the roots (Lakshmanan *et al.*, 2014; Tadych and White, 2019). Endophytic microorganisms occupy various host plant tissues without expressing any apparent disease expression and can be isolated from the seeds, stems, leaves, flowers and fruits (Roze *et al.*, 2011; Currie *et al.*, 2014). The relationship between endophytic fungi and their host plant is mutualistic. Plants provide settlement and nutrition to endophytic fungi. In return, endophytic fungi preserve host plants from biotic and abiotic stresses by delivering various secondary metabolites (Larriba *et al.*, 2015). So far, the main phylum of endophytic fungi that have been isolated is *Ascomycota*. While *Aspergillus* sp. is the most studied genus of endophytic fungi (Rustamova *et al.*, 2020).

The ethnobotanical information on herbal used for traditional medicine becomes an excellent source for isolating endophytes microbes (Lutfia *et al.*, 2020). *Aquilaria* species (agarwood) from the Thymelaeaceae family is one of the most valuable plants on earth and has been used in religious, aromatic and medicinal preparations for thousands of years (Wyn and Anak, 2010). Agarwood plants are found natively in Bangladesh, India, Indonesia, Malaysia, Myanmar and Vietnam (Hashim *et al*., 2016). It is a large evergreen tree growing over 15-30 m in height and 1.5-2.5 m in diameter and is able to produce fragrant agarwood resins when the wood is infected by pathogens or wounded (Hashim *et al*., 2016). *Aquilaria malaccensis* is a potential pharmacological plant due to its capacity to generate various secondary metabolites. *Aquilaria malaccensis* leaves extract has antioxidant (Hendra *et al*., 2016; Halim

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(1) 2023, pp. 55-62
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et al., 2022), antiviral against dengue virus (Masita *et al.*, 2020) and immunomodulatory potential (Batubara *et al*., 2021; Wahid *et al*., 2022; Yana *et al*., 2022). Furthermore, the stem has been referenced for anticancer activities (Hegde *et al*., 2018).

Several previous studies have isolated endophytic fungi from *A. malaccensis*. For example, Shoeb *et al*. (2010) isolated endophytic fungi from *A. malaccensis* stems, leaves and roots. However, the study did not identify the obtained fungi and instead concentrated on the ability of endophytic fungi to produce secondary metabolites and their potential as antibacterial and identifying the compounds. Other studies from India have been reported by Mochahari *et al*. (2020), which isolated endophytic fungi from the stems and wood chips of juvenile *A. malaccensis*. Premalatha and Kalra (2013) isolated and identified endophytic fungi on wood chips of *A. malaccensis.* Chhipa and Kaushik (2017) observed the diversity of fungi and bacteria in trees and soil around *A. malaccensis* plants. Only one study identifying the endophyte of *A. malaccensis* from Aceh, Indonesia, has been reported resulting in only *Fusarium* sp. (Zulfendi *et al.*, 2019). The reports lead us to believe that different regions and plant parts have different endophytic fungi associated with *A. malaccensis*. This research is then aimed at isolating and morphologically characterizing the endophytic fungi from *A. malaccensis*.

MATERIALS AND METHODS

Sample collection

The plant materials used in this study were taken from the stems and leaves of three healthy three-year-old *A. malaccensis* trees obtained from the Sawit Sari Research Station, Faculty of Biology, Universitas Gadjah Mada, Sleman Regency, Yogyakarta, Indonesia. The stems and leaves were then stored in sterile zipper bags and placed inside the icebox.

Isolation and culturing of endophytic fungi

Samples were exposed to surface sterilization following the procedure described by Katoch and Pull (2017) with modification. First, samples were washed using running tap water for 10 min to remove dust and dirt. Then they were cut to a size of around 1 cm, followed by sterilization using 75% ethanol for 1 min, followed by 3% sodium hypochlorite solution for 1 min, and then rinsed with sterile water three times for 1 min each and dried with sterile paper. The sterile samples were put into PDA media which already contained chloramphenicol to suppress bacterial contamination. In each petri dish, one piece of the sample was placed. Samples were then incubated at 28 °C for 7-14 days until the mycelium grew. The mycelium that grew closest to where the sample was put on the media was considered an endophytic fungal isolate. The inoculation of endophytic fungi from these petri dishes was carried out by transferring hyphal tips to fresh PDA plates to yield pure culture for identification. The sterile water used for rinsing was poured into a sterile medium to confirm the successful sterilization. No growth of fungi, indicating no contamination on the surface of the samples.

Morphological identification

Morphological characters were recorded for identifying the endophytic fungi, including growth pattern, hyphae, colony color, surface texture, margin character, conidiophore and conidia formation. The collected data was then compared and checked using the description in the Pictorial Atlas of Soil and Seed Fungi 3rd Edition, Fungi Identification Guidebook (Watanabe, 2010).

RESULTS AND DISCUSSION

The isolation of leaves and stems of *A. malaccensis* resulted in a total of 14 endophytic fungi detected, including 5 endophytic fungi discovered from the leaves and 9 endophytic fungi from the stem. This number is higher than that discovered by Shoeb *et al*. (2010), who isolated the endophytic fungi from *A. malaccensis* in Sylhet, Bangladesh, acquiring just two strains from the leaves and four from the stems. Moreover, Mochahari *et al.* (2020) reported the discovery of *Alternaria* sp., *Curvularia* sp., *Rhizopus* sp. and *Sterilia* sp. from the stems of juvenile *A. malaccensis*. Each plant component will yield a varied number of endophytic fungi isolates, revealing that the fungi are the most broadly dispersed species in the plant (Fitriarni and Kasiamdari , 2018).

The isolation and identification of endophytic fungi from *A. malaccensis* revealed more prevalent white fungal colonies, with only two black colonies detected (Figure 1). Based on microscopic characterization, six isolates i.e., LA2, LA3, LA4, LA5, SA1, SA2, SA3, SA6 and SA9 are enable to generate spores (Figure 2). The results showed that isolate LA2 is comparable to *Neopestalotiopsis* sp., LA3 to *Aspergillus* sp., LA4 to *Aspergillus* sp., LA5 to *Arthrinium* sp., SA1 to *Curvularia* sp., SA2 to *Podospora* sp., SA3 to *Mucor* sp., SA6 to *Verticillium* sp. and SA9 to *Mucor* sp. (Table 1).

Characteristics of LA2 on culture medium showed white mycelium, with an aspect cottony. This isolate formed fusiform conidia in four ellipsoid septa, the apical and basal cells were conical and hyaline, while the cell before the basal cell was lighter than the third and fourth cells. The conidia had a basal appendix and two to three filiform appendages. These characteristics are similar to the description for the genus *Neopestalotiopsis* sp. (Intriago-Reyna *et al.*, 2021). Characteristics of LA3 and LA4 on culture medium showed white mycelium with a fluffy texture. These isolates formed globose conidia. These characteristics are similar to the description for the genus *Aspergillus* sp. (Ezeonuegbu *et al*., 2022). Characteristics of LA5 on culture medium showed white mycelium. Conidia are brown, smooth to granular and globose to elongate ellipsoids in surface view. These characteristics are similar to the description for the genus *Arthrinium* sp. (Kwon *et al*., 2021).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.221535

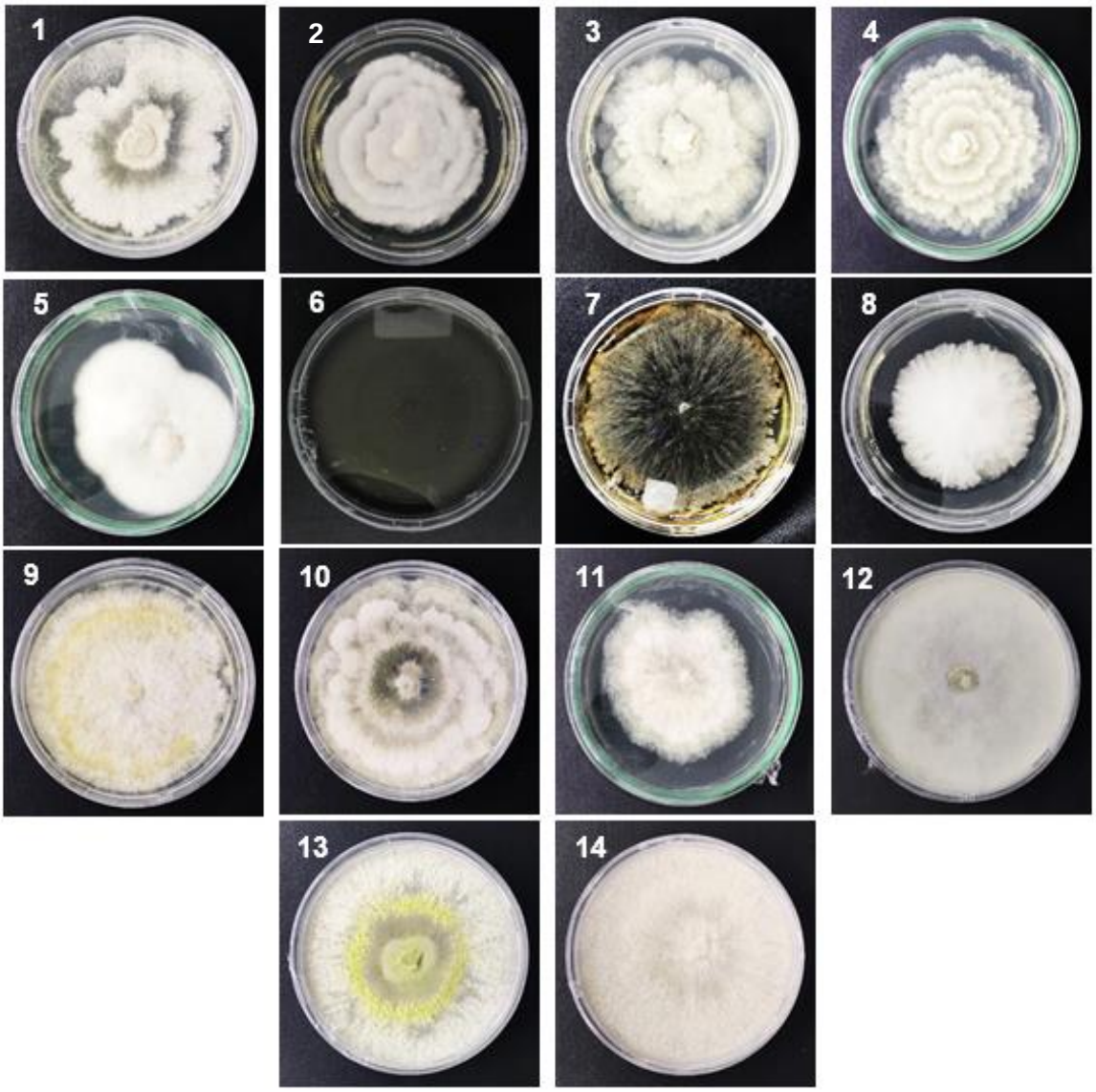

Figure 1: Colony morphology variations of endophytic fungi from *Aquilaria malaccensis* on PDA at day seven. Number 1 to 5 from leaves (isolate 1: LA1, isolate 2: LA2, isolate 3: LA3, isolate 4: LA4, isolate 5: LA5). Number 6 to 14 from stems (isolate 6: SA1, isolate 7: SA2, isolate 8: SA3, isolate 9: SA4, isolate 10: SA5, isolate 11: SA6, isolate 12: SA7, isolate 13: SA8, isolate 14: SA9).

Characteristics of SA1 on culture medium showed brown mycelium with a cottony texture. Conidia are ellipsoidal, gently curved and brown with 3 septa. These characteristics are similar to the description for the genus *Curvularia* sp. (Leewijit *et al*., 2016). Characteristics of SA2 on culture medium showed brown mycelium with a cottony texture. Conidia are globose and brown. These characteristics are similar to the description for the genus *Podospora* sp. (Sun and Guo, 2012). Characteristics of SA3 and SA9 on culture medium showed white mycelium; the sporangia are globose. These characteristics are similar to the description for the genus *mucor* sp. (Dyląg *et al.*, 2019). Characteristics of SA6 on culture medium showed white colony and conidia are globose. These characteristics are similar to the description for the genus *Verticillium* sp. (Inderbitzin *et al.*, 2011).

In addition, isolates LA1, SA4, SA5, SA7 and SA8 were found to be absent of conidia production. These isolates are classified as mycelia sterile since they did not display anamorph or teleomorph forms throughout the observation procedure (Asman *et al.*, 2018). Furthermore, isolates LA1, SA4, SA5, SA7 and SA8 do not create conidiophores or conidia in this investigation. This condition may be due to poor growth medium conditions for conidia formation, or the fungi may take longer to form reproductive structures. Chen *et al*. (2013) discovered the

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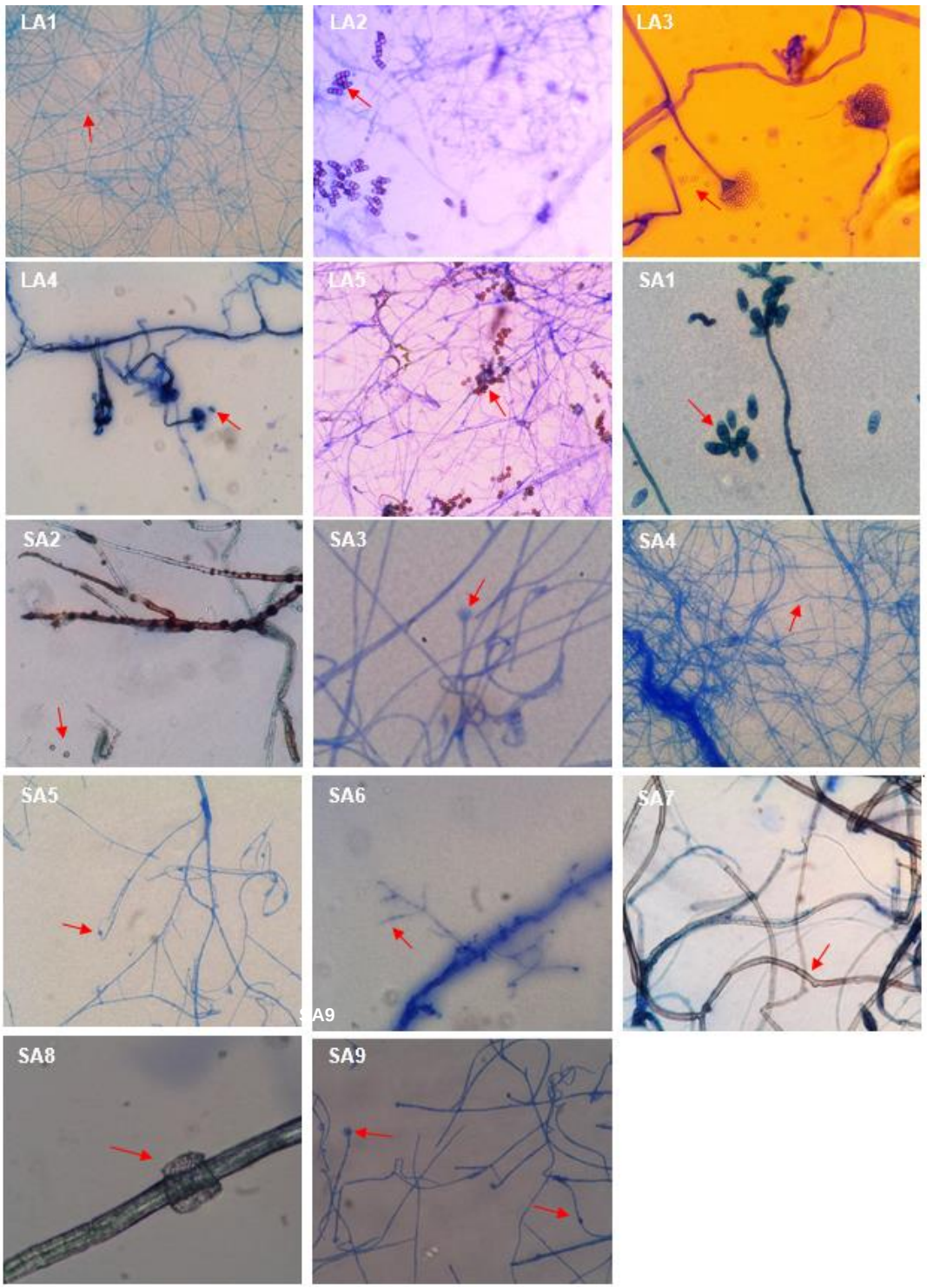

Figure 2: Microscopic characteristics of endophytic fungi isolated from *Aquilaria malaccensis* observed under a light microscope with 400× magnification (LA1: hyphae of mycelia sterile, LA2: conidia of *Neopestalotiopsis* sp., LA3: conidia of *Aspergillus* sp., LA4: conidia *Aspergillus* sp., LA5: conidia *Arthrinium* sp., SA1: conidia of *Curvularia* sp., SA2: conidia of *Podospora* sp., SA3: sporangia of *Mucor* sp., SA4: hyphae of mycelia sterile, SA5: chlamydospores of mycelia sterile, SA6: conidia of *Verticillium* sp., SA7: hyphae of mycelia sterile, SA8: chlamydospores of mycelia sterile, SA9: sporangia of *Mucor* sp.).

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Table 1: The description of macroscopic and microscopic characteristics of each endophytic fungi isolated from *Aquilaria malaccensis*.

Genus	Isolate code	Plant part	Colony morphology	Hyphae	Conidia character
Mycellia sterilia	LA1	Leaf	Color: white, Reverse: yellowish, Shape: irregular, Texture: cottony.	Septate	-
Neopestalotiopsis sp.	LA2	Leaf	Color: white, Reverse: yellowish, Shape: irregular, Texture: cottony.	Septate	Conidia are fusiform conidia in four ellipsoid septa. The apical and basal cells were conical and hyaline, while the cell before the basal cell was lighter than the third and fourth cells.
Aspergillus sp.	LA3	Leaf	Color: white, Reverse: yellowish, Shape: irregular, Texture: fluffy.	Septa	Conidia are globose.
Aspergillus sp.	LA4	Leaf	Color: white, Reverse: yellowish, Shape: irregular, Texture: fluffy.	Septa	Conidia are globose.
Arthrinium sp.	LA5	Leaf	Color: white, Reverse: yellowish, Shape: irregular, Texture: smooth.	Septa	Conidia are brown, smooth to granular and globose to elongate ellipsoid in surface view.
Curvularia sp.	SA1	Stem	Color: brown, Reverse: black, Shape: circular, Texture: cottony.	Septae	Conidia are ellipsoidal, gently curved and brown with 3 septa.
Podospora sp.	SA2	Stem	Color: brown, Reverse: black, Shape: irregular, Texture: fluffy.	Septa	Conidia are globose and brown in color.
Mucor sp.	SA3	Stem	Color: white with a yellow ring, Reverse: brown, Shape: circular, Texture: cottony.	Non-septate	Sporangia are globose.
Mycellia sterilia	SA4	Stem	Color: white, Reverse: brown, Shape: irregular, Texture: cottony.	Non-septate	-
Mycellia sterilia	SA5	Stem	Color: white, Reverse: yellowish, Shape: irregular, Texture: fluffy.	Non-septate	-
Verticillium sp.	SA6	Stem	Color: white, Reverse: yellowish, Shape: irregular, Texture: fluffy.	Septate	Conidia are globose.
Mycellia sterilia	SA7	Stem	Color: white, Reverse: greenish, Shape: circular, Texture: cottony.	Septate	-
Mycellia sterilia	SA8	Stem	Color: white with yellow ring, Reverse: yellowish, Shape: circular, Texture: cottony.	Septate	-
Mucor sp.	SA9	Stem	Color: white, Reverse: yellowish, Shape: circular, Texture: cottony.	Non-septate	Sporangia are globose.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

same issue, finding no reproductive structure in isolates of endophytic fungi from *Dendrobium* sp. species plants by morphological studies under a microscope. As a result, only molecular identification can confirm the species of these endophytic fungi.

Endophytic fungi from the phylum *Ascomycota* were the most prevalent endophytic fungi isolated from diverse plants, followed by *Basidiomycota* (Rana *et al.*, 2019). The endophytic fungus observed in this study had previously been described; for example, *Neopestalotiopsis* sp. was detected on the tropical mangrove trees in Krabi, Thailand (Kumar *et al.*, 2019). *Curvularia* sp. and *Fusarium* sp. were identified on *A. malaccensis* wood chips in Assam, India (Premalatha and Kalra, 2013). Mycelia sterile were found on palm trees located in Bangkok, Thailand (Song *et al*., 2016). *Arthrinium* sp. and *Fusarium* sp. were discovered on the stem of *A. subintegra* in Chiang Mai, Thailand (Monggoot *et al*., 2017). *Podospora* sp. was isolated from the Kenyan medicinal plant *Laggera alata* (Matasyoh *et al*., 2011). In addition, *Muccor* sp. was discovered on *A. beccariana* and *A. microcarpa* from Brunei Darussalam (Mohammad *et al.*, 2021).

The investigation of the fungal endophytic population of plants is undoubtedly a significant difficulty that science has gradually solved. The existence of these microorganisms within plant tissues is difficult to detect, hyphae are rarely visible and distinguishing features are limited (Rashmi *et al*., 2019). The endophytic community has conventionally been examined by isolating fungal strains from surface-sterilized plant tissues, with the target of recovering fungal strains present only in the plant's inner. As a result, standard culture media are utilized, with changes as needed, such as the inclusion of a more significant proportion (typically double) of water in the medium to avoid an osmotic chock and promote the access of exploratory hyphae (Murphy *et al*., 2018).

Plants evolved in reaction to their microbial surroundings (Li *et al*., 2016; Murat *et al*., 2018). Over 407 million years, plant-microbial interactions have been observed (Hardoim *et al*., 2015). Plants are inhabited internally by a wide range of fungi, which may cross the endodermis barrier without being targeted by plant immune signals, migrating from the root cortex to the vascular system and subsequently surviving as endophytes in plant organs (Leach *et al*., 2017; Hassani, 2018). Endophytic fungi are transmitted horizontally or vertically in plant tissues, whereas horizontal transmission occurs when vegetative propagules or spores are produced by the endophyte and spread to the plant population through the air or via some vector, and vertical transmission consists of the fungi being transferred to the plant progeny via seeds (Aly *et al*., 2011; Lugtenberg *et al.*, 2016). The endophytic fungal community in plants is greatly affected by plant part, plant exudates (Sasse *et al*., 2018), age, climate (Thiergart *et al*., 2020), nutrient balance, geographical location (Vincent *et al*., 2016) and season (Ray *et al*., 2019).

CONCLUSION

The research findings suggest that the number of endophytic fungi found in various organs differs. Not all endophytic fungi isolates that grow can produce sexual phages. Nine of the fourteen endophytes which can produce conidia were identified as *Neopestalotiopsis* sp., *Aspergillus* sp., *Arthrinium* sp., *Curvularia* sp., *Podospora* sp., *Mucor* sp. and *Verticillium* sp.

ACKNOWLEDGEMENTS

This study was funded by LPDP (Lembaga Pengelola Dana Pendidikan). The authors thank the University of Gadjah Mada (UGM), the Microbiology laboratory of the Research Center for Biotechnology UGM, the technicians and those who have helped during this study.

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