Diversity and antioxidant activity of endophytic fungi isolated from salam (Syzygium polyanthum)

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Submission date: 14-Jun-2023 08:50PM (UTC+0700)
Submission ID: 2115965803
File name: 14355-Article_Text-1084244-1-10-20230612.pdf (781.33K)
Word count: 7803
Character count: 44636

BIODIVERSITAS
Volume 24, Number 5, May 2023
Pages: 3051-3062

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240561

Diversity and antioxidant activity of endophytic fungi isolated from *salam* (*Syzygium polyanthum*)

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Manuscript received: 6 April 2023. Revision accepted: 29 May 2023.

Abstract. *Widjajanti H, Elfita, Sari MT, Hidayati N, Hariani PL, Setiawan A. 2023. Diversity and antioxidant activity of endophytic fungi isolated from* salam *(Syzygium polyanthum). Biodiversitas 24: 3051-3062.* Salam (*Syzygium polyanthum* (Wight) Walp.) is a medicinal plant from the family Myrtaceae. The leaves are used as a spice and treat diseases such as diabetes mellitus, hypertension, ulcers, diarrhea, gastritis, and skin. This study aimed to determine endophytic fungi's diversity and antioxidant activity in the *S. polyanthum* plant. In addition, the distribution of endophytic fungi found on *S. polyanthum* was compared to that of previously reported endophytic fungi on other species of *Syzygium*. The distribution pattern of these endophytic fungi is needed to determine the presence of potential endophytic fungi that can live in host plants of the same genus. The endophytic fungi were isolated from fresh root bark, stem bark, and leaf tissues of *S. polyanthum*. The endophytic fungi were identified morphologically to determine their level of diversity; their extract was then tested for their antioxidant activity using the DPPH method. The extract that showed the highest antioxidant activity was identified molecularly. A total of 18 endophytic fungi were obtained from *S. polyanthum*, including seven isolates from root bark (HSA1–HSA7), four isolates from stem bark (HSB1–HSB4), and seven isolates from leaves (HSD1–HSD7). The morphological identification showed seven genera scattered in all investigated parts: *Trichoderma*, *Penicillium*, *Aspergillus*, *Pythium*, *Papulaspora*, *Pythiogeton*, and *Clonostachys*. The HSD5 isolate showed the best antioxidant activity, and the molecular identification confirmed this isolate as *Clonostachys rosea*. The comparison of the distribution of endophytic fungi isolated from *S. polyanthum* to endophytic fungi isolated from the host genus *Syzygium* showed that endophytic fungi of three genera, namely *Clonostachys*, *Papulaspora*, and *Pythiogeton*, are specifically found on *S. polyanthum*. In addition, those of four other genera, namely *Trichoderma*, *Aspergillus*, *Penicillium*, and *Pythium*, are found on other species of *Syzygium*.

Keywords: Antioxidant, diversity, endophytic fungi, *Syzygium polyanthum*

INTRODUCTION

The leaves of salam (*Syzygium polyanthum* (Wight) Walp.) are commonly used as a spice because they have a distinctive aroma (Nordin et al. 2017). Moreover, water boiled with *S. polyanthum* is often used as a traditional medicine to treat diseases such as diabetes mellitus, hypertension, gastritis, and skin. In addition, secondary metabolites, such as terpenoids, flavonoids, niacin, and tannins, can reduce levels of uric acid and triglycerides, reduce pain, and act as natural antioxidants (Gutiérrez-Del-río et al. 2021; Abu Hajleh et al. 2022; Michalak et al. 2022; Oktiansyah et al. 2022).

Natural antioxidants effectively inhibit free radicals in treating several diseases, such as cancer, hypertension, and neurodegenerative diseases (Forman and Zhang 2021; Collins et al. 2022). Therefore, consuming foods or substances containing antioxidant compounds is important. There is currently a strong drive to find abundant, readily available, and efficient sources of natural antioxidants, to replace synthetic antioxidants to reduce the damage to our cells (Zehiroglu and Ozturk 2019; Flieger et al. 2021; Munteanu and Apetrei 2021). Medicinal plants cultivation in Indonesia faces many obstacles in terms of production, such as the unprofessional implementation of medicinal plant cultivation activities (Jadid et al. 2020; Salmerón-Manzano et al. 2020; Moraes et al. 2021; Vaou et al. 2021), the farmers' inability to maintain the quality of medicinal plants, and the medicinal plant industry lack of attention to apply scientific research results in their product developments (Astutik et al. 2019; Mohammadi and Saghaian 2022). Therefore, to overcome these challenges, endophytic fungi biotechnology has become the focus of research to find new antioxidant compounds and their diversity to balance the ecosystem.

Diversity is vital for life on Earth because it provides all kinds of natural resources needed for survival. Each species plays an important role in the ecosystem (Han et al. 2022). The loss of key species can disrupt ecosystems, negatively impacting all living things and destroying these ecosystems (Brema et al. 2022; Shivanna 2022). The diversity of endophytic fungi in a plant can be very important information to identify species with great potential in the medicinal world (Niu et al. 2022; Tripathi et al. 2022;

Wang et al. 2022). The same species of endophytic fungi in different medicinal plants can be used as a source of new raw materials for the health sector so that drug needs can be met and become agents for preventing disease resistance (Adeleke and Babalola 2021; Silva et al. 2022). Therefore, information on the diversity of endophytic fungal biotechnology is needed.

Endophytic fungi are a critical tool in biotechnology because they produce secondary metabolites and can be propagated quickly (Elfita et al. 2012; Alam et al. 2021; Oktiansyah et al. 2023a). Some endophytic fungi produce certain phytochemical compounds that are also produced by their host plants due to their interactions with host plants through coevolutionary processes (Ju et al. 2022; Xia et al. 2022). Secondary metabolites derived from endophytic fungi are the most relevant discoveries for new drug substances. Researchers have shown that the compounds derived from endophytic fungi have complex and unique chemical structures, meaning that they are either distinct novel molecules or similar to those produced by their hosts (Caruso et al. 2022; Ortega et al. 2021; Zheng et al. 2021; Elfita et al. 2023). The medicinal plants' demand in the world is projected to increase significantly, given the growing function of medicinal plants and the pattern of public awareness of the more natural medicines. The medicinal plant *S. polyanthum* has a great opportunity to obtain new drugs from its endophytic fungi to enhance the immune response and prevent multi-drug resistance. This study aimed to find opportunities to obtain secondary metabolites with high antioxidant activity from various endophytic fungi isolated from *S. polyanthum*. In addition, the distribution of endophytic fungi found in *S. polyanthum* was compared to the distribution of previously reported endophytic fungi in other species of *Syzygium*.

MATERIALS AND METHODS

Plant sample

Syzygium polyanthum was collected from Lahat District, South Sumatra (16 August 2022; Longitude: 103.129165°; Latitude: -4.095762°). Plants have been identified in the Plant Systematics Laboratory, Universitas Sriwijaya, number 328/UN9.1.7/4/EP/2022. Isolation of endophytic fungi used healthy and fresh plant tissues.

Isolation of endophytic fungi

Plant organs (root bark, stem bark, and leaves) were washed with running water. Next, plant organs were soaked in 70% alcohol (±3 minutes), rinsed with sterile distilled water (±1 minute), then immersed in 3% sodium hypochlorite (NaOCl) (±1 minute). Sterilized organs were aseptically cut into small pieces. Plant organs were inoculated to a petri dish containing Potato Dextrose Agar medium and then incubated for 3-14 days at room temperature (26°C). Observations were made every day until the fungi were seen. The colonies growing around the organs with different characteristics (color, size, and texture) were then purified. Purification was done by transferring the colonies to a petri dish containing PDA media and then incubating them at room temperature for 3 days. Finally, the purified colonies were transferred to the culture medium (Elfita et al. 2022).

Identification of endophytic fungi morphologically

Macroscopic and microscopic characteristics were used to identify endophytic fungi. Observations of macroscopic characteristics included: the color of the colony surface and the reverse side, the texture of the colony (cotton, grain, powder, slimy), the presence of exudate droplets, the presence of radial lines, and concentric circles. The slide culture method analyzes microscopic characteristics by observing hyphae, spores, color, and other specific properties under a microscope up to 1000× magnification. The appeared characters were then compared with relevant references for fungi identification (Pitt et al. 2013; Watanabe 2010; Walsh et al. 2018)

Molecular identification of endophytic fungi

Isolates of endophytic fungi with the best antioxidant activity were followed identification molecularly based on the (ITS) DNA (rDNA) gene amplification process used ITS1 and ITS4 primers. The sequences were then entered into the Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Next, sample sequences and databases were aligned using the CLUSTAL W in the MEGA11 program and reconstructed the phylogenetic tree using the Neighbor-joining with a bootstrap value of 1000 (Tamura et al. 2021).

Extraction and cultivation

The endophytic fungi isolated from *S. polyanthum* were cultivated by placing 5 blocks (5 mm diameter) of pure culture agar into 2 culture bottles containing Potato Dextrose Agar medium with a volume of 300 mL. Cultures were incubated for 30 days at room temperature in static conditions. After the incubation, the mycelia and media were separated using filter paper. Furthermore, ethyl acetate was added to the media (1:1) and extracted. Finally, the ethyl acetate extract was separated to be evaporated using a rotary evaporator, and the extract was concentrated using an oven at 45°C (Hapida et al. 2021; Oktiansyah et al. 2023b).

Antioxidant activity test

The antioxidant activity analysis used the DPPH method. First, each endophytic fungi extract was dissolved in methanol into a concentration of 1,000, 500, 250, 125, 62.5, 31.25, 15.625 µg/mL (three repetitions). Next, each concentration (0.2 mL) was added to 3.8 mL of 0.5 mM DPPH solution. The mixture was homogenized and left in a dark tube for 30 minutes. The absorbance level was measured using a UV/Vis spectrophotometer at λmax 517 nm. In this test, ascorbic acid was used as the standard antioxidant. Next, the antioxidant activity was calculated through the percentage of DPPH absorption inhibition and the value of IC_{50} (Abbas et al. 2021).

$$\% \text{ Inhibition} = \frac{A_k - A_s}{A_s}$$

A_k = Absorbance of control
A_s = Absorbance of samples

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

There were 18 isolates of endophytic fungi isolated from *S. polyanthum*, consisting of seven isolates from root bark (HSA1-HSA7), four isolates from stem bark (HSB1-HSB4), and seven leaves isolates (HSD1-HSD7). These endophytic fungal isolates showed distinctive and diverse macroscopic and microscopic characteristics (Figures 1, 2, and 3). The colony colors observed were white, gray, yellow, green, and black. The results of observing the characteristics of endophytic fungal isolates from each organ macroscopically and microscopically are shown in Tables 1, 2, 4, 5, 7, and 8.

Tables 1 and 2 describe the characteristics of the endophytic fungi colonies from the root bark of *S. polyanthum* in each isolate. Furthermore, a total 3 genera of endophytic fungi were found, namely *Trichoderma* (3 isolates: HSA1, HSA3, HSA6), *Aspergillus* (3 isolates: HSA2, HSA4, HSA5), and *Penicillium* (HSA7). Based on the appearance of morphological characteristics (macroscopic and microscopic), 7 isolates of the endophytic fungi from the root bark of *S. polyanthum* were identified.

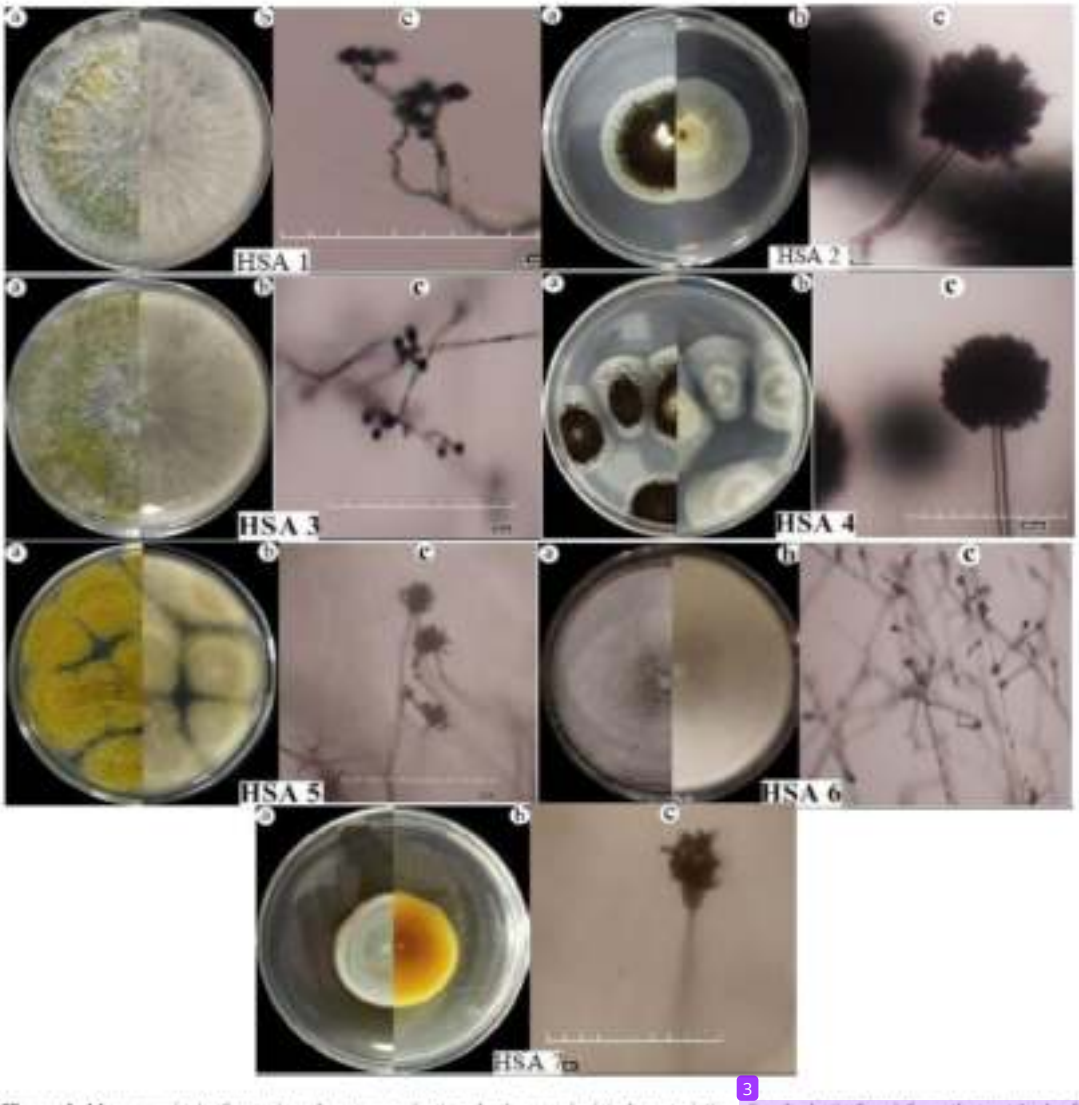

Figure 1. Macroscopic (a: front view, b: reverse view) and microscopic (c) characteristics of endophytic fungi from the root bark of *Syzygium polyanthum*

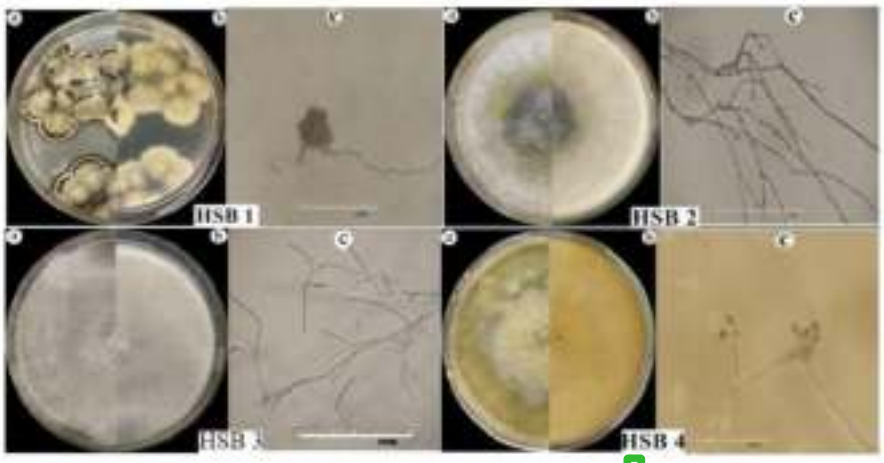

Figure 2. Macroscopic (a: front view, b: reverse view) and microscopic (c) characteristics of endophytic fungi from the stem bark of *Syzygium polyanthum*

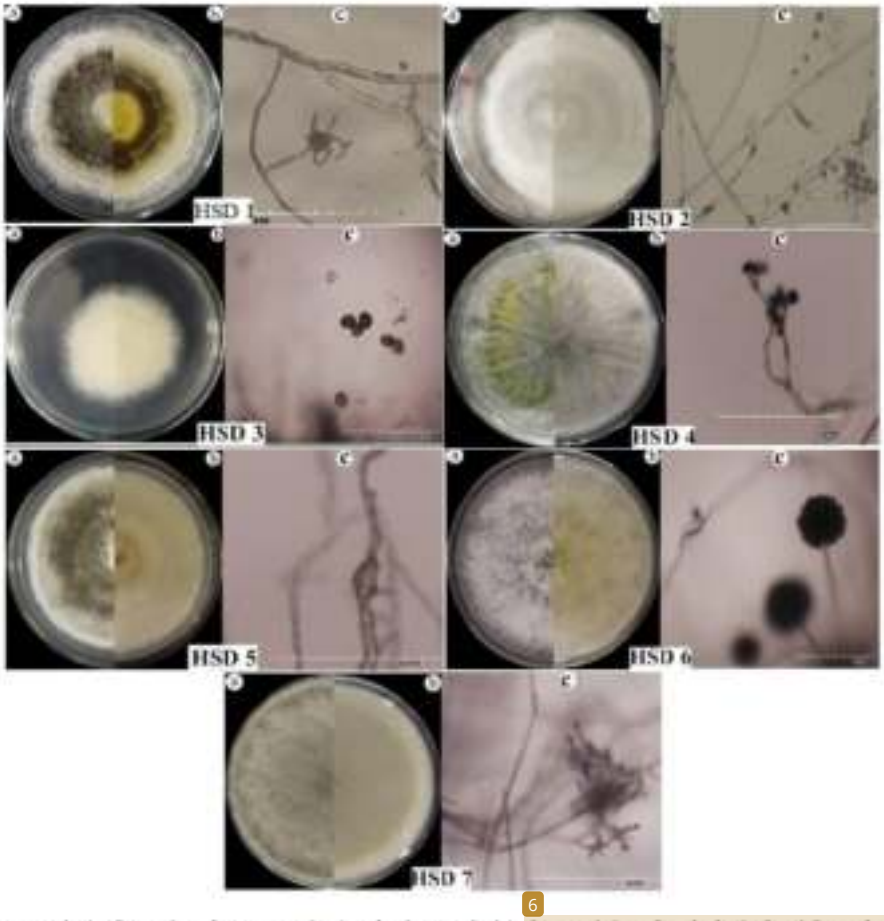

Figure 3. Macroscopic (a: front view, b: reverse view) and microscopic (c) characteristics of endophytic fungi from the leaves of *Syzygium polyanthum*

Table 3 explains extracts of endophytic fungi isolated from the root bark of *S. polyanthum*, which exhibit very strong antioxidant activity (IC_{50} <20 µg/mL), equivalent to host plants and ascorbic acid, namely isolate HSA1. Several other endophytic fungi extracts showed strong (IC_{50}: 20-100 µg/mL: isolates HSA4, HSA5, HSA6, and HSA7), moderate (IC_{50}: 101-500 µg/mL: isolates HSA3), and weak (IC_{50} > 500 µg/mL: isolate HSA2).

Table 4 and Table 5 describe the morphological characteristics of the endophytic fungi colonies from the stem bark of *S. polyanthum* in each isolate. There were 3 genera of endophytic fungi found, namely *Penicillium* (3 isolates: HSB1), *Pythium* (2 isolates: HSB2, HSB3), and *Trichoderma* (HSB4). Based on the morphological characteristics that appeared, 4 isolates of the endophytic fungi from the stem bark of *S. polyanthum* were identified.

Table 6 showed extracts of endophytic fungi isolated from the stem bark of *S. polyanthum*, which exhibit very strong antioxidant activity (IC_{50} <20 µg/mL), equivalent to host plants and ascorbic acid, namely isolate HSB1. Several other endophytic fungi extracts showed strong (IC_{50}: 20-100 µg/mL: HSB2, HSB4) and weak (IC_{50} >500 µg/mL: isolate HSB3) antioxidant activity.

Table 1. Colony characteristics of endophytic fungi from the root bark of *Syzygium polyanthum*

Code	Surface colony	Reverse colony	Structure	Elevation	Pattern	Exudate drops	Radial line	Concentric circle
HSA 1	White with yellowish green tint	White	Cottony	Umbonate	Radiate	-	√	-
HSA 2	Black greenish with whitish tint	Tan to brown	Velvety	Umbonate	Radiate	-	-	√
HSA 3	White with yellowish green tint	Brownish white	Cottony	Rugose	Zonate	-	√	-
HSA 4	Black with white border	White to cream	Powdery	Umbonate	Spread	-	-	-
HSA 5	Yellow	Yellowish to tan	Powdery	Umbonate	Spread	-	-	-
HSA 6	Whitish grey	Grayish brown	Cottony	Umbonate	Radiate	-	-	-
HSA 7	Grayish green	Yellowish brown	Velvety	Umbonate	Zonate	-	√	√

Table 2. Microscopic characteristics of endophytic fungi from the root bark of *Syzygium polyanthum*

Isolate	Spore	Shape	Hyphae	Characteristic	Species of identification
HSA 1	Conidia	Subglobose	Septate	Conidiophores hyaline, erect, branched, bearing spore masses apically at irregularly disposed of phialides; phialides short and thick.	*Trichoderma pseudokoningii*
HSA 2	Conidia	Subglobose	Septate	Phialides radiate around antire vesicles and are biseriate, with the metulae twice as long as the phialides.	*Aspergillus niger*
HSA 3	Conidia	Subglobose	Septate	Conidiophores hyaline, erect, branched, bearing pore masses apically at irregularly, was disposed of phialides; phialides short and thick.	*Trichoderma pseudokoningii*
HSA 4	Conidia	Subglobose	Septate	Phialides radiate around antire vesicles and are biseriate, with the metulae twice as long as the phialides.	*Aspergillus niger*
HSA 5	Conidia	Globose	Septate	The conidiophores are long, and when fully mature, the walls are characteristically rough/spiny, especially at the tips.	*Aspergillus flavus*
HSA 6	Conidia	Subglobose	Septate	Conidiophores hyaline, erect, branched, bearing spore masses are apically at phialides.	*Trichoderma koningii*
HSA 7	Conidia	SubGlobose	Septate	Conidiophores hyaline, developed from aerial hyphae, erect, slightly rough	*Penicillium nigricans*

Table 3. Antioxidant Activity (IC50 value) of Endophytic fungi extract isolated from the root bark of *Syzygium polyanthum*

Sample	Extract	Species	Antioxidant activity IC_{50} (µg/mL)
Host plant	Methanol extract of the host plant		21.00 ***
Endophytic fungi	HSA 1	*Trichoderma pseudokoningii*	19.68 ****
	HSA 2	*Aspergillus niger*	509.29 *
	HSA 3	*Trichoderma pseudokoningii*	122.25 **
	HSA 4	*Aspergilus niger*	23.34 ***
	HSA 5	*Aspergilus flavus*	53.35 ***
	HSA 6	*Trichoderma koningii*	25.99 ***
	HSA 7	*Penicillium nigricans*	29.96 ***
Positive control	Ascorbic acid		10.08 ****

Note: Antioxidant activity IC_{50} (µg/mL): ****very strong < 20 µg/mL ***strong < 100 µg/mL; **moderate 100-500 µg/mL; * weak > 500 µg/mL

Table 7 and Table 6 described the characteristics of the endophytic fungi colonies from the leaves of *S. polyanthum* in each isolate. There were 6 genera of endophytic fungi found, namely *Papulaspora* (1 isolate: HSD1), *Trichoderma* (2 isolates: HSD2, HSD4), *Pythiogeton* (1 isolate: HSD3), *Clonostachys* (1 isolate: HSD5), *Aspergillus* (1 isolate: HSD6) and *Pythium* (1 isolate: HSD7). Based on the morphological characteristics, 7 isolates of the endophytic fungi from leaves of *S. polyanthum* were identified.

Table 9 showed several extracts of endophytic fungi isolated from the leaves of *S. polyanthum*, which exhibit very strong antioxidant activity (IC_{50} <20 µg/mL), equivalent to host plants and ascorbic acid, namely isolates HSD2, HSD4, HSD5, and HSD7. Several other endophytic fungi extracts showed strong (IC_{50}: 20-100 µg/mL: HSD1) and moderate (IC_{50}: 101-500 µg/mL: HSD3 and HSD6) antioxidant activity.

Table 4. Colony characteristic of endophytic fungi from the stem bark of *Syzygium polyanthum*

Code	Surface colony	Reverse colony	Structure	Elevation	Pattern	Exudate drops	Radial line	Concentric circle
HSB 1	Yellowish brown	Yellowish brown	Powdery	Umbonate	Spread	-	-	-
HSB 2	White with yellow	Brownie	Cottony	Umbonate	Zonate	-	√	√
HSB 3	White	White	Cottony	Rugose	Zonate	-	-	-
HSB 4	Pale brown	White with yellow	Cottony	Umbonate	Radiate	-	√	√

Table 5. Microscopic characteristics of endophytic fungi from the stem bark of *Syzygium polyanthum*

Isolate	Spore	Shape	Hyphae	Characteristic	Species of identification
HSB 1	Conidia	Cylindrical	Septate	Conidiophores hyaline, erect, branched penicillately at the apexes with verticillate metula and terminal phialides.	*Penicillium corylophilum*
HSB 2	Sporangia	Globose	Septate	Sporangia lobate, terminal or intercalary, rarely discharging zoospores, mostly functioned as conidia germinated by germ tubes.	*Pythium dissimile*
HSB 3	Sporangia	Cylindrical	Septate	Hyphae often show dendroid branching, sometimes slightly swollen, often directly connected with sexual organs. No zoospore dischatged.	*Pythium indigoferae*
HSB 4	Conidia	Subglobose	Septate	Conidiophores hyaline, erect, branched, bearing spore masses apically at phialides; phialides tapering toward the apex.	*Trichoderma koningii*

Table 6. Antioxidant activity (IC_{50} value) of endophytic fungi extract isolated from the stem bark of *Syzygium polyanthum*

Sample	Extract	Species	Antioxidant activity IC_{50} (µg/mL)
Host plant	Ethanol extract of the host plant		18,07 ****
Endophytic fungi	HSB 1	*Penicillium corylophilum*	16,56 ****
	HSB 2	*Pythium dissimile*	23,35 ***
	HSB 3	*Pythium indigoferae*	508,47 *
	HSB 4	*Trichoderma koningii*	60,39 ***
Positive control	Ascorbic acid		10,083 ****

Note: antioxidant activity IC50 (µg/mL): ****very strong < 20 µg/mL, ***strong < 100 µg/mL; **moderate 100-500 µg/mL; * weak > 500 µg/mL

Table 7. Colony characteristic of endophytic fungi from the leaves of *Syzygium polyanthum*

Code	Surface colony	Reverse colony	Structure	Elevation	Pattern	Exudate drops	Radial line	Concentric circle
HSD 1	Grayish brown	White soft brown	Cottony	Rugose	Radiate	-	√	√
HSD 2	White	White	Cottony	Umbonate	Zonate	-	-	√
HSD 3	White	White	Cottony	Umbonate	Zonate	-	-	√
HSD 4	White with yellowish green tint	Brownish white	Cottony	Rugose	Zonate	-	√	-
HSD 5	Grayish	White	Cottony	Umbonate	Radiate	-	√	√
HSD 6	White	White	Cottony	Umbonate	Radiate	-	-	√
HSD 7	Grayish	White	Cottony	Umbonate	Zonate	-	-	-

Table 8. Microscopic characteristics of endophytic fungi from the leaves of *Syzygium polyanthum*

Isolate	Spore	Shape	Hyphae	Characteristic	Species of Identification
HSD 1	Sporangia	Globose	Septate	Conidiophores for phialoconida hyaline, erect, simple, rarely branched.	*Papulaspora nishigaharanus*
HSD 2	Sporangia	Globose	Septate	Conidiophores hyaline, phialides short and thick, globose	*Trichoderma harzianum*
HSD 3	Sporangia	Globose	Septate	Sporangia cylindrical, globose, ellipsoidal, or irregular in shape.	*Pythiogeton ramosum*
HSD 4	Conidia	Subglubose	Septate	Conidiophores hyline, erect, branched, bearing pore masses, was apically at irregularly disposed phialides. Phialides short and thick.	*Trichoderma pseudokoningii*
HSD 5	Sporangia	Subglobose	Septate	Sporangia terminal or intercalary, lobate, subglobose or irregular.	*Clonostachys rosea*
HSD 6	Conidia	Globose	Septate	The conidiophores are long, and when fully mature, the cells are characteristically rough/spiny.	*Aspergillus* sp.
HSD 7	Lobate	Glubose	Septate	Sporangia lobate, terminal was intercalary, and zoospores rarely or not discharged.	*Pythium periplocum*

Table 9. Antioxidant activity (IC_{50} value) of extracts of endophytic fungi isolated from the leaves of *Syzygium polyanthum*

Sample	Extract	Species	Antioxidant activity IC_{50} (µg/mL)
Host plant	Methanol extract of the host plant		11,85****
Endophytic fungi	HSD1	*Papulaspora nishigaharanus*	25,42***
	HSD2	*Trichoderma harzianum*	12,09****
	HSD3	*Pythiogeton ramosum*	208,84 **
	HSD4	*Trichoderma pseudokoningii*	17,12****
	HSD5	*Clonostachys rosea*	11,80****
	HSD6	*Aspergillus sp.*	206,45 **
	HSD7	*Pylsium periplocum*	12,47****
Positive control	Ascorbic acid		10,08 ****

Note: antioxidant activity IC50 (µg/mL): ****very strong < 20 µg/mL ***strong < 100 µg/mL; **moderate 100-500 µg/mL; * weak > 500 µg/mL

Antioxidant activity of endophytic fungi extract from *S. polyanthum*

Endophytic fungi isolated from *S. polyanthum* extracted using ethyl acetate had the potential as antioxidants. Its antioxidant activity showed an IC_{50} included in the very strong, strong, medium, and weak categories. In addition, six endophytic fungi extracts showed very strong antioxidant activity, as seen in Figure 4.

Figure 4 shows several endophytic fungi extracts that exhibit very strong antioxidant activity (IC_{50} <20 µg/mL), equivalent to host plants and ascorbic acid, namely isolates HSA1, HSB1, HSD2, HSD4, HSD5, and HSD7. HSD5 was selected as the most potential endophytic fungi because it had antioxidant activity equivalent to ascorbic acid and had the highest extract weight; Furthermore, HSD5 was followed by molecular identification.

Molecular identification of endophytic fungi

HSD5 isolated from *S. polyanthum* was identified molecularly. Its antioxidant activity showed the best results compared to other extracts. The results can be seen in Figure 5 as a phylogenetic tree. The sequence of ITS rDNA isolate HSD5 was as follows:

TGGGGGGCATTCTACTGATCTGAGGTCACCTTGGAAG
TTGGGGGTTTAACGGCAGGGGCTCGTCGCTCTCCGATGC
GGAATATCACTACTTCGCAGGGGAGGCCACGACGGGTCC
GCCACTAGATTTAGGGGCCGGCCGTCCCTCGCGGGCTTT
GGCCGATCCCCAACACCACGCCCTAGGGGCATGAGGGTT
GAAATGACGCTCAGACAGGCATGCCCGCCAGAATACTGG
CGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGA
ATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTT
CTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAG
TTTTTATTTATTGTAAAAACTACTCAGAAGATTCCAAA
ATAAAACAAGAGTTAAGTTTCCTAGGCGGGCGCCTGATC
CGGGGCACACGAGGCGCCCGGGGCAATCCCGCCGAAGCA
ACAGTAGGTATGTTCACATGGGTTTGGGAGTTGTAAACT
CGGTAATGATCCCTCCGCTGGTTCACCAACGGAGACCTT
GTTACGACTTTTTACTTCAAA.

Based on the molecular test, HSD5 isolate was identified as *Clonostachys rosea*.

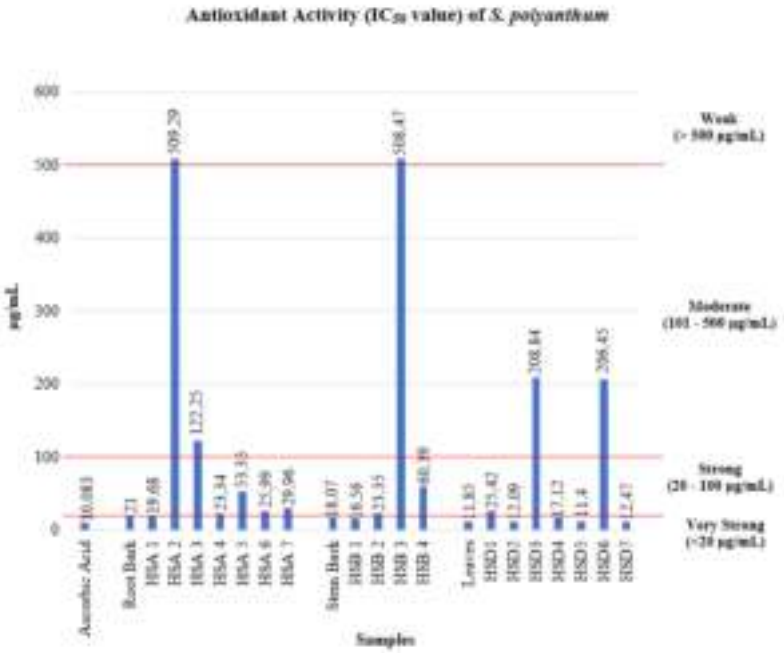

Figure 4. Antioxidant Activity (IC_{50} value) of Endophytic fungi Extract Isolated from *Syzygium polyanthum*. Note: (i) HSA1-HSA7 = Endophytic fungal colonies isolated from the root bark of *S. polyanthum*; (ii) HSB1-HSB4 = Endophytic fungal colonies isolated from the stem bark of *S.* polyanthum; (iii) HSD1-HSD7 = Endophytic fungal colonies isolated from leaves of *S. polyanthum*

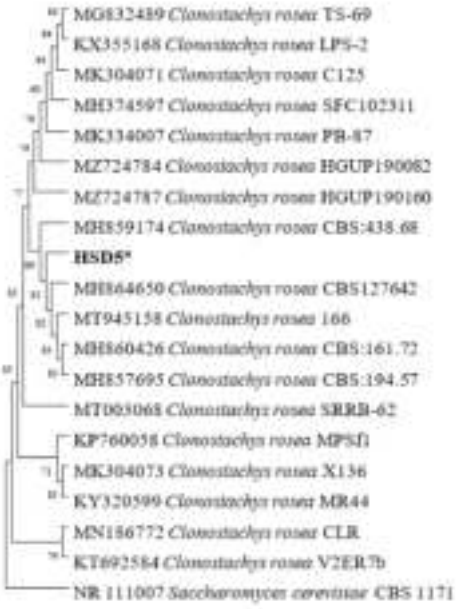

Figure 5. The phylogenetic tree of HSD5 isolates (signed *) was reconstructed using the Neighbor-Joining (bootstrap value = 1000)

Distribution of endophytic fungi isolated from the genus of *Syzygium*

Based on a literature study on endophytic fungi isolated from several plants of the genus *Syzygium* (Figure 4), several genera of endophytic fungi are scattered in several plants of the genus *Syzygium*. In this study, the genus *Clonostachys*, which had very strong antioxidant activity and the highest extract yield, was an endophytic fungus only found in *S. polyanthum*. That indicated *S. polyanthum* was a specific host for endophytic fungi of the genus *Clonostachys*.

Figure 6 shows the distribution of endophytic fungi isolated from plants of the genus *Syzygium*. A genus of endophytic fungi was found in several plants from the genus *Syzygium*. The genus of *Aspergillus* found in *Syzygium jambos* (L.) Alston, *Syzygium zeylanicum* (L.) DC., *Syzygium aqueum* (Burm.fil.) Alston, and *S. polyanthum*; genus of *Penicillium* (found in *Syzygium malaccense* (L.) Merr. & L.M.Perry, *S. zeylanicum*, *S. aqueum*, and *S. polyanthum*); genus of *Trichoderma* (found in *S. zeylanicum*, *S. aqueum*, and *S. polyanthum*); genus of *Phialemonium* (found in *S. malaccense* and *S. zeylanicum*); genus of *Acremonium* (found in *S. jambos* and *S. zeylanicum*); genus of *Chaetomium* (found in *S. zeylanicum* and *S. aqueum*); genus of *Pythium* (found in *S. malaccense*, *S. zeylanicum*, and *S. polyanthum*); and genus of *Madurella* as well as *Poaceascoma* (found in *S. malaccense* and *S. jambos*).

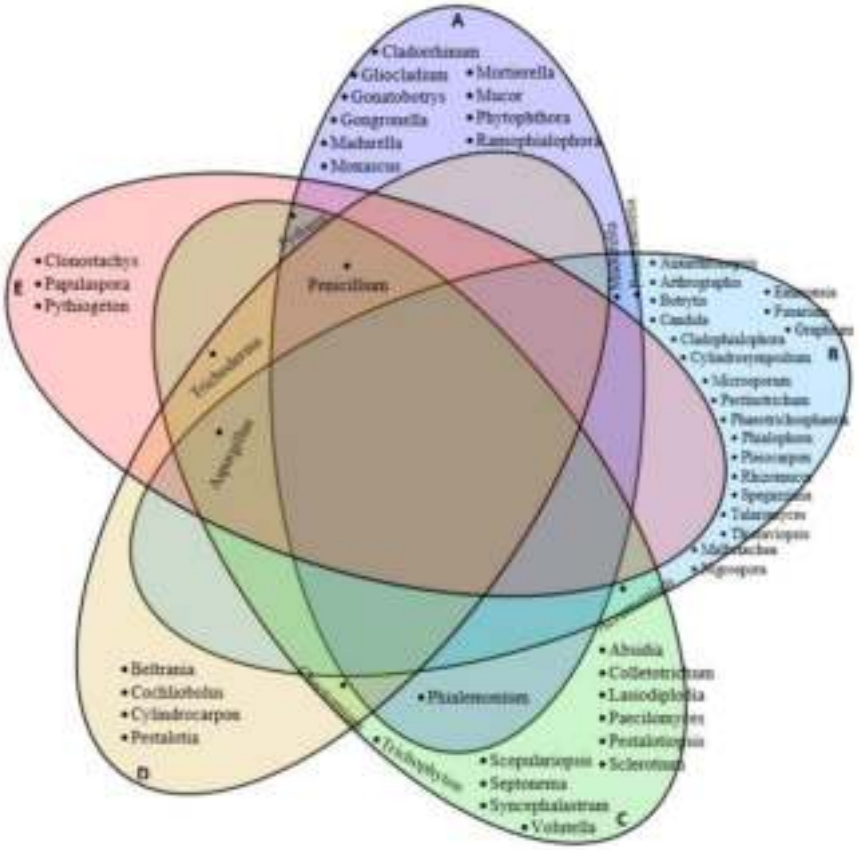

Figure 6. Distribution of endophytic fungi isolated from the genus of *Syzygium*. A. *Syzygium malaccense* (Hapida et al. 2021); B. *Syzygium jambos* (Aini et al. 2022); C. *Syzygium zeylanicum* (Syarifah et al. 2021); D. *Syzygium aqueum* (Habisukan et al. 2021); E. *Syzygium polyanthum*

Discussion

In this study, 18 endophytic fungi isolates were identified from *S. polyanthum* belonging to 7 genera (i.e., *Trichoderma*, *Penicillium*, *Aspergillus*, *Pythium*, *Papulaspora*, *Pythiogeton*, and *Clonostachys*). Furthermore, *Trichoderma* was found in three organs of *S. polyanthum*. Based on the morphological identification, the endophytic fungi found in the root and stem bark were identified as *Trichoderma koningii*, and the endophytic fungi found in the root bark and leaves were identified as *Trichoderma humatum* and *Trichoderma pseudokoningii*. This finding reveals that the endophytic fungi of the genus *Trichoderma* do not have site specificity for living in plant tissues, as found by several researchers (Fang et al. 2019; Morán-Diez et al. 2021; Andrzejak and Janowska 2022; Tyśkiewicz et al. 2022; Oktiansyah et al. 2023c). However, previous studies have reported that endophytic fungi of the genus *Trichoderma* are found in specific organs of their host plants. Figure 6 confirms that endophytic fungi from the genus *Trichoderma* are also found in *S. zeylanicum* and *S. aqueum*. The findings of this study indicate that endophytic fungi from the genus *Trichoderma* can survive in their hosts' anatomical structure and physiological conditions differences.

On the other hand, the genus *Clonostachys* is a genus that is only found in *S. polyanthum* (Figure 6), namely *Clonostachys rosea*, which was isolated from the leaves of *S. polyanthum*. Based on scientific information, this species has never been found on *S. polyanthum* before. Furthermore, *C. rosea* is a promising saprophytic fungus that belongs to the phylum Ascomycota. This fungus lives in many types of habitats, with the highest frequency found in the soil. Therefore, as an excellent mycoparasite, *C. rosea* exhibits strong biological control capabilities against many fungal, nematode, and insect-plant pathogens (Piombo et al. 2023; Song et al. 2024; Venkatesan et al. 2023). This behavior is according to the activation of several mechanisms, such as secreted enzymes to degrade the cell wall, antifungal secondary metabolites production, and plant defense systems induction. Besides having significant biocontrol activity, *C. rosea* also functions in the biodegradation of plastic waste, biotransformation of

bioactive compounds, and source of bioenergy (Sun et al. 2020; El-Gendi et al. 2022; Geiger et al. 2022; Kiruba and Saeid 2022; Kapesa-Ndacnou et al. 2023). The findings in this study complement the benefits provided by *C. rosea* that were previously studied. In addition, the antioxidant activity shown by the endophytic fungi of *C. rosea* extract was very strong (IC_{50} < 20 µg/mL) with an extract weight of 1.6 grams.

The four genera identified from *S. polyanthum*, namely *Trichoderma* (HSA1), *Aspergillus* (HSA4), *Penicillium* (HSB1), and *Pythium* (HSD7), are also found on other species of the genus *Syzygium*. These genera's antioxidant activity falls in the very strong and strong category. Genera *Trichoderma*, *Penicillium*, and *Pythium* have very strong antioxidant activity (IC_{50} <20 µg/mL) with extract weights of 1.1, 1, and 1 g, respectively, and the genus *Aspergillus* shows strong antioxidant activity (IC_{50} < 100 µg/mL) with an extract weight of 1.2 g. Similar antioxidant activity is shown by the four genera found in *S. zeylanicum*. In addition, Syarifah et al. (2021) reported that *Trichoderma koningii* (ZT2), *Aspergillus niduluns* (ZL9), *Penicillium citrinum* (ZL6), and *Pythium sp.* (ZL1) isolated from *S. zeylanicum* have very strong antioxidant activity with extract weights of 6.8, 4.6, 6.7, and 4.7 g, respectively. That indicates endophytic fungi of the same genus or species show different levels of antioxidant activities and extract weights used. This finding reveals that it is possible to isolate endophytic fungi from plants of the same genus to increase their overall yield and satisfy the raw materials demands with very strong antioxidant activity. The higher yield value indicates that the resulting extract is greater. Therefore, obtaining yield in large quantities requires several parameters, such as extraction methods, amount of biomass, environmental conditions (pH, temperature, humidity), and extraction time (Liu et al. 2022; Monagas et al. 2022; Nortjie et al. 2022). The selection of pre-treatment and processing methods affects the reduction time for extraction, an increase in yield, the quality of biological compounds, and energy reduction (Zhang et al. 2018; Sandhu et al. 2021; Putra et al. 2022). For example, drying impacts the metabolites of heat-sensitive components, and the process can also contribute to improving the conservation of bioactive compounds against oxidation, enzymatic activity, and putrefactive bacteria and allows cell destruction. Furthermore, this drying process can affect the results of the bioactivity of the extract (Gąsecka et al. 2020; Benjamin et al. 2022; Zubia et al. 2023).

The antioxidant activity of the methanol extracts of *S. polyanthum* root, bark, and leaf bark showed strong (IC_{50} < 100 µg/mL) and very strong (IC_{50} <20 µg/mL) activities, almost equivalent to standard antioxidants (positive control). The strong bioactivity of this methanol extract is closely related to its chemical composition. *S. polyanthum* contains terpenoids, flavonoids, niacin, and tannins (Ismail and Wan Ahmad 2019; Oktiansyah et al. 2022). That means there is a synergy between the compounds in the *S. polyanthum* extract, which causes its antioxidant activity to be very strong. Studies have revealed that these secondary metabolites have antioxidant activity (Ahmed et al. 2022; Hayat et al. 2020; M. Kumar et al. 2021; Sufeiman et al. 2021). Therefore, this study recommends that the leaves of *S. polyanthum* are better used as traditional medicine than other parts because it shows the best antioxidant activity. In addition, this very strong antioxidant activity is also shown by its endophytic fungi extract.

The endophytic fungus extracts isolated from *S. polyanthum* also had activity equivalent to that of its host. Endophytic fungi extracts HSA1, HSB1, HSD2, HSD4, HSD5, and HSD7 have very strong antioxidant activity (IC_{50} < 20 µg/mL) compared to other endophytic fungi extracts. The HSD5 isolate extract showed the closest IC_{50} value to the positive control (ascorbic acid) IC_{50} value. Based on molecular tests, the HSD5 isolate was identified as *C. rosea*.

This study found that the extract produced by the fungus *C. rosea* had very strong antioxidant activity. Various studies have shown that *C. rosea* contains clonostalactam, glioperazine, verticillin, benzoic acid, and several monoterpenoid groups (Han et al. 2020; Mascarin et al. 2022). These secondary metabolites show a variety of structures, such as the presence, number, and position of substitutional hydroxyl groups and the length of saturated side chains that give this compound its ability as an antioxidant (Miklasińska-Majdanik et al. 2018; Bouarab-Chibane et al. 2019; Kumar and Goel 2019; Putra 2012). Furthermore, benzoic acid can reduce and withstand free radical oxidative stress throughout physiological and biochemical reaction series (Abd Elhamid et al. 2022; Kupnik et al. 2023; Serventi et al. 2023). According to its biological effect, the endophytic fungi *C. rosea* isolated from *S. polyanthum* leaves can be used as a promising source of natural products for medicinal purposes.

ACKNOWLEDGEMENTS

The authors thank the Universitas Sriwijaya, which funded this research throughout *Skema Penelitian Unggulan Profesi* number 0111/UN9.3.1/SK/2022 dated 28 April 2022.

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