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Abstract. *Styrax benzoin* is a native tree to Indonesia, particularly in North Sumatra. This plant species produces benzoin resin, which is beneficial for medicinal treatments, hence its commercial value. Endophytic fungi help produce bioactive metabolites and contribute to resin production. However, the diversity of endophytic fungal species from *S. benzoin* grown in North Sumatra remained largely unexplored. This study aims to determine the distribution and diversity of culturable endophytic fungi from two *kemenyan*-producing locations in North Sumatra, Simalungun and North Tapanuli, as well as their tree part origin. A total of 7 and 8 endophytic fungal species were obtained from barks, stems, and/or leaves of *S. benzoin* grown in Simalungun and North Tapanuli, respectively, and identified by internal transcribed spacer sequence analysis. Endophytic fungi from North Tapanuli showed higher diversity, with a Shannon-Wiener index of 2.31 than those from Simalungun (1.95). Morisita-Horn similarity indices for bark-stem, stem-leaf, and bark-leaf were 0.47, 0.08, and 0, respectively, hinting at organ-specificity colonization. This study offers insights into the diversity of endophytic fungi isolated from *S. benzoin* which may contribute to future improvement of benzoin resin production.

1. Introduction

Benzoin resin is balsamic resin exudated from *Styrax* trees [1]. This genus is widely but disjunct Amphi-Pacific tropical distributed [2]. Two of well-known benzoin-producing *Styrax* species, particularly from South East Asia, are *S. tonkinesis* and *S. benzoin*. *S. tonkinensis* are found naturally and largely cultivated in Laos and Vietnam for wood and non-timber products [3], whereas *S. benzoin* is widely cultivated in North Sumatra province, Indonesia, particularly in North Tapanuli and Simalungun districts [4]. Benzoin resin produced by *S. benzoin*, which is called *kemenyan* in native Indonesian, is widely used for incense and medicinal purposes. Although the trade of benzoin resin is not well documented and outdated, partly due to naming inconsistency or misclassification [1], *kemenyan* production in North Sumatra had reached 8332 tons from 23,068 ha plantation in 2018 [4].

Benzoin resin was reported to have anti-inflammatory properties [5], antioxidant and antibacterial activities [6, 3], emphasizing its value in medical treatment. Its derivatives are also used in cosmetics, perfume, and other pharmaceutical products, making them economically valuable [5, 7]. Benzoin resin from *S. benzoin* as non-timber products with such value should be explored. Not only will it help

conserve forests and prevent illegal logging [8], but it will also benefit forest communities as a household income contributor. Therefore, thorough investigations on *S. benzoin* ecology and biological interactions are imperative for the future improvement of benzoin resin production.

All plants, particularly tropical trees, may host endophytic fungi in a natural ecosystem in which endophytic fungal diversity is higher than those from the plant in other regions [9]. Endophytic fungi grow and colonize host plant inner tissue and have mutual relations with their host [10]. These fungi can be found on the roots, stems, and leaves and do not damage their host, separating them from pathogenic fungi [11, 12]. On the contrary, these fungi increase host resistance and provide protection from pathogenic fungi, pests and predators [13, 14]. The idiosyncratic relationship between endophytic fungi and their respective host plants even expands to their metabolite production. It was reported that both hosts and endophytes might produce similar metabolites [15]. Furthermore, endophytic fungi have contended as the producer of extracted bioactive compounds from medicinal plants [16]. Such a noteworthy relationship urges further inquiry on the interaction between endophytic fungi and their host plants, particularly plants that are sources of commercially valuable compounds like *S. benzoin*.

Microbial endophytes from *S. benzoin* were reported to have antibacterial properties [17]. It was proposed that bioactive metabolites extracted from plants were actually fungal metabolites [18]. Considering the *S. benzoin* high value as a *kemenyan*-producing tree and the idiosyncratic relationship between endophytic fungi and their hosts, investigation on endophytic fungi that reside within *S. benzoin* is indispensable. However, studies on this subject are still fairly limited. Therefore, this study aimed to explore and observe the diversity of culturable endophytic fungal species isolated from barks, stems, and leaves of *S. benzoin* grown in benzoin-producing locations in North Sumatra as well as diversity measures endophytic fungal communities from *S. benzoin* and another *kemenyan*-producing *S. sumatrana*.

2. Materials and Methods

2.1. Chemicals and plant materials

Chemicals that were used in this study were analytical grade whenever possible, which include alcohol, sodium hypochlorite (NaOCl) 4%, potassium hydroxide (KOH) 10%, hydrogen chloride (HCl), glycerol 20% (Merck, Germany), Potato Dextrose Agar (PDA), and Malt Extract Agar (MEA) (Himedia, India). Samples from *S. benzoin* trees were collected from plantations in North Tapanuli (1°56' 40.626" N latitude and 99° 0' 51.663" E longitude) and Simalungun (2° 43' 33.012" N latitude and 98° 56' 18.432" E longitude) Regency, North Sumatra Province, Indonesia. Seven and 13 trees with breast-high diameters between 15 to 25 cm were selected from North Tapanuli and Simalungun plantations, respectively. In addition, at least two of three tree parts (leaf, stem and/or bark) from each tree that appeared to be symptomless were randomly collected.

2.2. Culturable endophytic fungi isolation

Samples were washed and rinsed following methods described in [19] and [20] to sterilize the surface of tree parts. Inner tissue of each sample was obtained after removing the outer layer aseptically. It was then cut into 5 x 5 mm thin pieces before being implanted on an agar isolation medium. PDA, yeast dextrose agar (YDA), yeast malt extract agar (YMA) and Pachlewksi (P5) media were used for isolation media [21]. Antibiotic chloramphenicol (100 mg/L) was added to suppress bacterial growth. Successful surface sterilization and endophytic fungal isolates were determined as described in [19] and [20]. During the incubation period, fungal colony development was observed. Fungal colonies grown from tissue fragments were cut, isolated, and subcultured on a fresh agar of the same isolation media. These cultures were then subjected to the identification.

2.3. DNA extraction and identification

Seven-day-old mycelial culture on potato dextrose broth (PBD) was used for genomic DNA extraction with DNA Wizard Kit (Promega, USA) as stated in the manufacturer's direction. ITS region of fungal DNA was amplified with ITS1 and ITS4 primer pair [22] and Go Taq® Green Master Mix (Promega,

USA). Amplified targeted DNA was visualized on 1.2% m/v agarose gel. Purified PCR products were Sanger-sequenced (First Base Sequencing Service, Singapore). BLASTn was used to find similarities in National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/).

2.4. Data analysis

Diversity was measured using the Shannon-Wiener index (H'), which was calculated by the following equation [23]:

$$H' = \sum PiInPi,\ Pi = Ni/Nt$$

where Ni is the number of isolates that belong to the i-th genus and Nt represents the total number of isolates in a group of interest (tree part or location). Genus relative abundance in tree parts was calculated as the percentage of the said genus abundance divided by total abundance present in each tree part [23]. Morisita-Horn similarity index on endophytic fungal communities between two locations and between *S. benzoin* and *S. sumatrana* [24] was calculated using EstimateS 9.1 software [25] to measure beta diversity (species-wise) between communities, where closer values to 0 indicate lower overlap and/or similarity between communities, and vice versa, closer values to 1 indicate higher overlap and/or similarity.

3. Results and Discussion

3.1. Endophytic fungal assemblage

A total of 20 endophytic fungal isolates from 12 species were successfully isolated from *S. benzoin.* Colonies of representative isolates are presented in Figure 1. Seven and 13 isolates from 7 and 8 species were obtained from Simalungun and North Tapanuli districts, respectively (Table 1). Four out of 7 isolates from Simalungun were obtained from leaf, whereas most isolates (7 out of 13) from North Tapanuli were isolated from the stem.

All fungal isolates were identified as species that belong to the Ascomycota division. A similar observation was reported on culturable endophytic fungi that were discovered from another *kemenyan*-producing tree, *Styrax sumatrana* [20]. Furthermore, four Ascomycota families were obtained in this study, namely Sporocadaceae, Diaporthaceae, Nectriaceae, and Hypocreaceae (Table 1). A total of 7 genera belonging to these families were isolated. Except for *Neopestalotiopsis formicarum*, *Fusarium solani*, *Pestalotiopsis* sp. and *Pestalotiopsis microspore*, the rest nine fungal species were only found once in either one of the tree parts (Table 1). Endophytic *Neopestalotiopsis*, *Pestalotiopsis* and *Fusarium* are well studied and have been obtained from various host plants, including medicinal ones [26]. *Pestalotiopsis microspore* isolated from such plants was reported to produce taxol, a chemotherapeutic compound used for cancer treatments [27].

Genus *Neopestalotiopsis* appeared to colonize various parts of *Styrax benzoin.* In this study, the genus, particularly *N. formicarum*, was found in bark and stem. This observation complemented a previous study that had obtained the genus in the fruit of *S. benzoin* grown in Aek Nauli, North Sumatra [26]. In addition, three species from this genus, namely *N. foedans, N. formicarum,* and *N. clavispora* were also found in the bark or leaf of *S. sumatrana* [20].

Genus *Diaporthe* was repeatedly isolated in this study (Table 1, Figure 2). The discovery of *Diaporthe* in stems and leaves in this study has added one more endophytic fungal genus to those previously found in *S. bemzoin* trees [26]. *Diaporthe* and endophytic *Neopestalotiopsis* were reported to produce antimicrobial and antioxidant compounds, eugenol [28], which may partly explain the medicinal properties of *kemenyan.* Furthermore, *Diaporthe* isolated from medicinal plant *Melodorum fruticosum* had an antioxidant activity that may be related to benzyl benzoate and benzyl cinnamate [29]. Interestingly, these compounds were also found as constituents of benzoin resin from *S. benzoin* [30].

Further investigations are imperative to inquire whether *Diaporthe* isolates that were obtained from *S. benzoin* also produce said compounds and whether their metabolites will support the argument that extracted bioactive compounds from *kemenyan*-producing *S. benzoin* are actually produced by

endophytic fungi [18]. Presuming that this preposition is accepted, future benzoin resin production can be improved by promoting the growth of these endophytic fungi. Therefore, understanding endophytic fungal assemblages and their interaction with host plants are foremost.

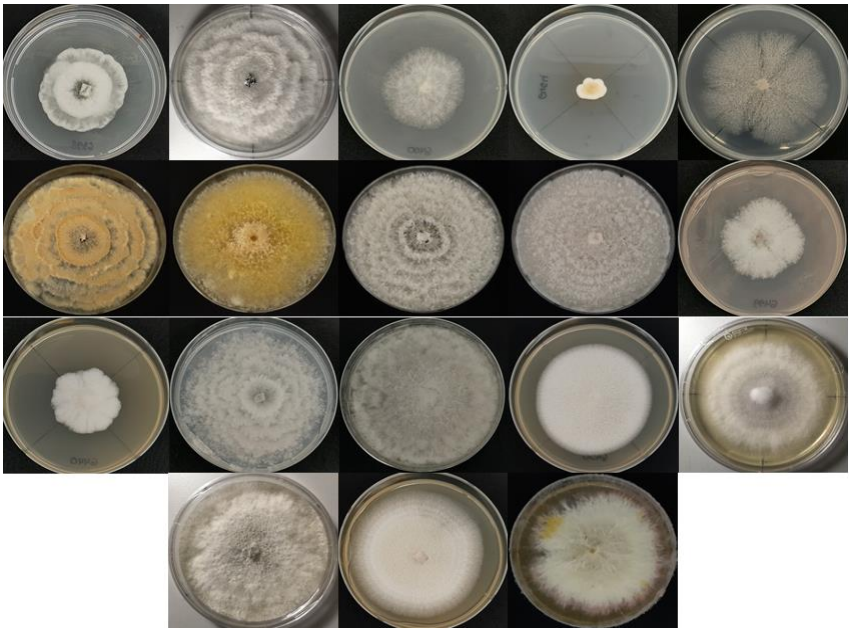

Figure 1. Representative fungal colonies that were isolated from *Styrax benzoin.* Fungal isolates were grown on their respective agar medium for 7 days at 25ºC.

Table 1. Culturable endophytic fungal species isolated from bark, stem, and leaf of *S. benzoin*.

	BLAST match results					Number of Isolates			
Closest Species	Similarity (%)	Accession Number	Division	Class	Family	Bark	Stem	Leaf	Total
Species from Simalungun district									
Clonostachys rosea	100	MN511326	Ascomycota	Sordariomycetes	Sporocadaceae	-	-	1	1
Pestalotiopsis microspora	100	MK120574	Ascomycota	Sordariomycetes	Sporocadaceae	1	-	-	1
Neofusicoccum parvum	100	MK334003	Ascomycota	Sordariomycetes	Sporocadaceae	-	1	-	1
Pestalotiopsis sp.	100	KP747695	Ascomycota	Sordariomycetes	Sporocadaceae	-	1	-	1
Diaporthe sp.	98	KU375708	Ascomycota	Sordariomycetes	Diaporthaceae	-	-	1	1
Fusarium graminearum	99	MN521508	Ascomycota	Sordariomycetes	Nectriaceae	-	-	1	1
Fusarium striatum	100	MH911354	Ascomycota	Sordariomycetes	Netriaceae	-	-	1	1
Subtotal						*1*	*2*	*4*	*7*
Species from North Tapanuli district									
Pestalotiopsis microspora	100	MK862237	Ascomycota	Sordariomycetes	Sporocadaceae	1	1	-	2
Acremonium sp.	100	MK651835	Ascomycota	Sordariomycetes	Hypocreaceae	1	-	-	1
Neopestalotiopsis formicarum	100	MN635622	Ascomycota	Sordariomycetes	Sporocadaceae	1	3	-	4
Diaporthe eucalyptorum	98	KX688169	Ascomycota	Sordariomycetes	Diaporthaceae	-	-	1	1
Diaporthe sp.	100	MH930430	Ascomycota	Sordariomycetes	Diaporthaceae	-	-	1	1
Fusarium solani	100	MG827182	Ascomycota	Sordariomycetes	Nectriaceae	-	1	1	2
Fusarium sp.	99	MN105567	Ascomycota	Sordariomycetes	Nectriaceae	-	1	-	1
Pestalotiopsis sp.	100	LC427210	Ascomycota	Sordariomycetes	Sporocadaceae	-	1	-	1
Subtotal						*3*	*7*	*3*	*13*
Total						**4**	**9**	**7**	**20**

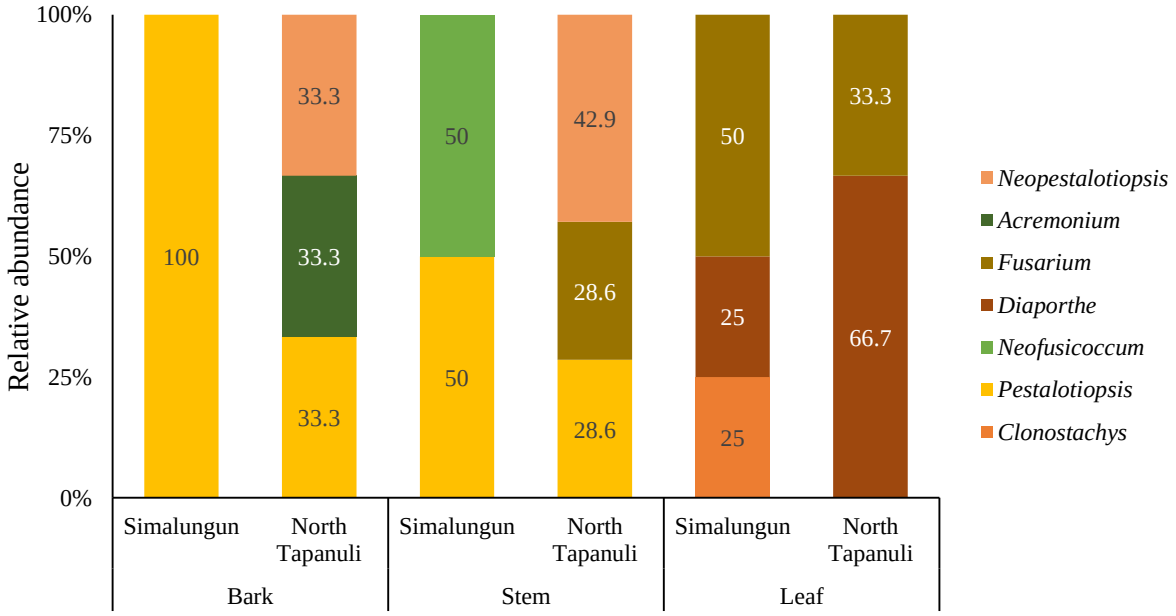

Figure 2. Relative abundance of endophytic fungal genera discovered in barks, stems, and leaves of *S. benzoin* grown in Simalungun and North Tapanuli.

The fungal assemblage revealed that tree parts of *S. benzoin* from North Tapanuli harbored more genera than those from Simalungun, except for the leaf (Figure 2). Compared to abiotic factors like climate or location, the assemblage of culturable endophytic fungi is more affected by the host tissue/organ where they reside [24, 31-33]. Therefore, different parts of the same tree and the same tree parts from the same tree species but grown in different locations (Simalungun and North Tapanuli) may harbor different fungal assemblages. The fungal assemblage from this study has added newly discovered species and genera of endophytic fungi found in newly added tree parts of *S. benzoin* (stem and leaf) from previously reported two genera from two tree parts, *Neopestalotiopsis* from fruit and *Schizophyllum* from the bark of *S. benzoin* [26].

3.2. Diversity of culturable endophytic fungi

Shannon-Wiener index was calculated to measure diversity for all samples from Simalungun and North Tapanuli. North Tapanuli appeared to have a higher diversity with an index value of 2.31 than 1.95 of Simalungun, which both are considered to have medium diversity [34]. In addition, a Morisita-Horn similarity index of 0.28 was obtained between Simalungun and North Tapanuli communities, which revealed rather a small overlap between these communities, *i.e.*, fungal communities harbored some species unique to the observed locations (Table 1). As previously stated, various environmental factors, such as climate, water availability [31], season and geographic locations [32], influence endophytic fungal communities, which was also reflected in diversity measures.

The same similarity index was also applied between pairs of tree parts, regardless of their locations. In the order from most to least similar, the bark-stem, stem-leaf, and bark-leaf indices were 0.47, 0.08, and 0, respectively. Thus, endophytic fungal communities in stem-leaf and bark-leaf had very little (one shared species, Table 1) and none overlap or shared species, respectively, between tree parts of each combination. This observation reflected that almost all species were unique to each tree part in these two pairs (Table 1). Such demonstration on organ specificity was also observed elsewhere [35]. Plant metabolites are secreted and distributed in a tissue-specific manner [36]. The micro-environment, which involves interaction with both plant tissue and other microbial endophytes in each organ, drives endophytic fungal communities' dynamics [37], which is consequently reflected in organ specificity [35]. The tissue/organ type of plant host has been repeatedly reported as a strong factor in determining

endophytic fungal community compositions [23, 31, 32, 38]. Out of the total seven species found in the bark and/or stem, two species were shared between these two organs, *i.e.*, the highest number of shared species among three tree part pairings. The tissue structures of stem and bark are different [39]. However, their proximity may explain the higher similarity index value. Further investigation is required to confirm whether the mycelia of the fungi inhabiting the stems extend to the barks and vice versa.

Table 2. Diversity of endophytic fungal communities in barks, stems, and leaves of *S. benzoin* and *S. sumatrana*.

Part of tree	Shannon-Wiener index (H')	
	S. benzoin	*S. sumatrana*[a]
Bark	1.04	**1.58**[b]
Stem	**1.67**	1.38
Leaf	**1.74**	0.37

Remarks: [a] Hidayat *et al.* (2021) [22].
[b] Higher values were indicated in bold.

An index value of 0.5 was obtained when the same similarity index was applied to observe the similarity between overall communities from this study (regardless of tree parts or locations) and from another *kemenyan*-producing tree, *S. sumatrana* [20] which were obtained using a similar approach. This result summarized that there were both shared and unique species between and to each host species. Although there were low similarities in fungal communities between locations as well as tree parts of *S. benzoin*, more species were shared between two *Styrax* species for overall communities. A closer look at each tree part, endophytic fungal communities in *S. benzoin* had higher diversity (Shannon-Wiener index) in stem and leaf communities than *S. sumatrana*. In contrast, higher diversity for bark communities was observed in *S. sumatrana* (Table 2). Among six communities in Table 2, only fungal communities in the leaves of *S. sumatrana* had low diversity (H' value < 1), whereas the rest had medium diversity [34].

In contrast to fungal communities in *S. sumatrana* which leaves had the lowest value of H', fungal communities in the leaves of *S. benzoin* had the highest H' value among their respective tree parts. Similarly, the lowest H' value for fungal communities in *S. benzoin* was found in bark, in which the highest H' value for *S. sumatrana* was observed. Thus, microbial endophytes were known to have specificity tendencies to each host plant species, as well as the organ/tissue of their respective hosts [26, 40]. Such niche diversification may have been rooted in complex interactions on a larger scale between host plant species and environment, and microscale between host tissue and fungal endophytes, as well as coexistence and competition among microbial endophytes [35]. Consequently, these interactions may have led to a selection process that favors host species- and organ-specific endophytic fungi [35] and is reflected in their community assemblages and diversity measures.

Information on the assemblage and diversity of endophytic fungi contribute to better understanding the microenvironment of medicinal plants. Previous studies on other medicinal plants have suggested that bioactive compounds harvested from medicinal plants were produced by their endophytic fungi [18, 29]. This finding urges further investigations on the metabolites that endophytic fungi from *S. benzoin* produce and their role in host plant metabolite production. Such information is imperative to improve benzoin production from *Styrax* trees and consequently increase their values.

4. Conclusion

Twenty endophytic fungal isolates from 12 species were obtained from barks, stems, and/or leaves of *S. benzoin* grown in Simalungun and North Tapanuli, North Sumatra, Indonesia. North Tapanuli had a higher diversity of endophytic fungi than Simalungun. Different diversity rankings for tree parts and similarities were observed between communities in *S. benzoin* and *S. sumatrana*, which indicate complex mechanisms underlying endophytic fungal assemblages. Although endophytic fungi obtained in this study are not as inclusive as they might have been with a metagenomic approach, the newly

discovered culturable fungi in this study offered insights into endophytic fungal communities within different plant organs commercially valuable *S. benzoin*. Relatively low numbers of shared species between two locations and even between tree parts hinted at the high specificity of fungal colonization. Further characterization of the obtained endophytic fungal isolates is necessary to improve benzoin resin production of *S. benzoin.*

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Authors' contribution
SA Faulina, A Hidayat, and M Turjaman designed and conducted the study, analyzed and interpreted data, provided materials, wrote, and finalized the manuscripts. WY Slamet and LM Rahayu conducted the experiments, computed data, and composed the manuscript. A Susilowati and D Elfiati helped in providing materials, writing, and overviewing the manuscripts. All authors have read, assessed, and validated the manuscript.