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Isolation and molecular identification of agarwood-inducing fungi and their virulence test using *Aquilaria* sp. seedlings

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Abstract. *Lukman, Dinarti D, Siregar UJ, Turjaman M, Sudarsono. 2023. Isolation and molecular identification of agarwood-inducing fungi and their virulence test using* Aquilaria *sp. seedlings. Biodiversitas 24: 140-148.* Agarwood is a lump of fragrant resin with a dark color and distinctive aroma from secondary metabolite products that accumulate in wood fibers produced from the genus *Aquilaria* and *Gyrinops*, a plant defense mechanism against damage caused by wounds and infection of specific fungal pathogens. The three main factors in producing agarwood sapwood are the presence of plants that have the potential to produce agarwood (family Thymelaceae) as a host, a supportive environment, and the presence of specific fungal pathogens. The isolation, identification, and virulence testing of local isolates must be made to get the correct agarwood-inducing pathogens without posing potential environmental problems. A total of five explants of *Aquilaria* sp. branches producing agarwood from the study site were used. This study aims to isolate local strains of fungi associated with agarwood formation from North Aceh regency, Aceh province, Indonesia, figure out their virulence against and ability to induce agarwood from seedlings of target trees, and determine their species identity. The experiments comprised isolation, macroscopic and microscopic identification, virulence tests, and species identification based on ITS sequences. The study resulted in five local Aceh isolates (LAI-1-LAI-5). Four isolates (LAI-1-LAI-4) can induce agarwood formation, and one isolate (LAI-5) is a white rot fungus. The ITS sequence analysis showed that LAI-1-LAI-4 were *Fusarium solani*, while two colonies taken from LAI-5 isolate were identified as *Ceriporia lacerata* or *Irpex laceratus* (LAI-5 colony#1), and *Daldinia eschscholtzii* (LAI-5 colony #3). Based on these findings, the LA-5 isolate was probably a mixture of the two white rot fungus isolates.

Keywords: Aceh, agarwood production, *Aquilaria*, *Fusarium solani*, ITS sequences

INTRODUCTION

Agarwood is a non-timber forest product with a distinct scent induced by wounding from pathogenic infection in the Thymeleaceae family, such as *Aquilaria* and *Gyrinops* species (Nguyen et al. 2014; Faizal et al. 2020; Mohammad et al. 2021; Du et al. 2022). The resins accumulated in the xylem of *Aquilaria sinensis* in response to the infection of pathogenic fungi are secondary metabolites produced through specific pathways (Gao et al. 2012). Agarwood is widely used in various industrial products, such as cosmetics and pharmacy, religious needs, and aromatherapy (Liu et al. 2013; Ye et al. 2016; Liu et al. 2017). Naturally, global agarwood production is limited while demand rises and exceeds the available supply. Therefore, countries producing agarwood could not fulfill the market need. The natural agarwood export quota from *Aquilaria malaccensis* from Indonesia was 151,725.00 kg in 2018, decreasing by 15% from the previous year. Meanwhile, natural and cultivated agarwood export in 2020 amounted to 101,000.00 kg, decreasing by 50,725.00 kg from 2018 (CITES Appendix II). Over-exploitation of natural agarwood, inappropriate harvesting practices, and lack of reforestation in natural habitats have partially caused the current reduction in natural agarwood yield.

The primary factors affecting agarwood production are the specific plant populations (i.e., members of the Thymelaceae family), the supportive environment conditions, and the availability of compatible microbes capable of inducing agarwood formation. Many researchers mentioned that agarwood is generated when the appropriate trees (*Aquilaria* sp. and *Gyrinops* sp.) are injured because of physical, lightning strikes, herding animals, and pathogenic attacks (Liu et al. 2013; Mohamedet al. 2014, Rasool and Mohamed 2016; Wu et al. 2017). Other researchers suggested that resin secretion from the defense reaction of *Gyrinops* sp. accumulated in the injured area and eventually became agarwood sapwood (Subasinghe and Hettiarachchi 2013). *Aquilaria* sp. and *Gyrinops* sp. are the most common plants producing agarwood in the Eastern part of Indonesia, such as the Papua, Sulawesi, Kalimantan, and Nusa Tenggara Islands. Meanwhile, *Aquilaria beccariana*, *A. malaccensis*, and *A. microcarpa* are commonly found in the Western part of Indonesia, such as in Sumatra (Lee et al. 2018). North Aceh regency, Aceh province, is one of Indonesia's natural agarwood producer areas.

In each region, there are different fungi associated with agarwood production. *Fusarium* sp., *Trichoderma* sp., and *Curvularia* sp. are fungal pathogens inducing agarwood from *A. Malaccensis* in India (Premalatha and Kalra 2013). Other fungi capable of inducing agarwood production include *Aspergillus* sp., *Penicillium, Fusarium, Lasiodiploidia, Chaetomium,* and *Botryodiplodia* sp. (Mohamed et al. 2014). Meanwhile, *Diplodia* sp., *Fusarium bulbiferum, F. lateritium, F. oxysporum, Pythium* sp., and *Trichoderma* sp. are known to infect *Aquilaria* sp.. However, not all fungi can induce agarwood production, and fungal species capable of inducing agarwood from *Aquilaria* sp. and *Gyrinops* sp. include *Fusarium* sp., *Lasiodiplodia* sp., *Penicillium* sp., and *Aspergillus* sp. (Sangareswari et al. 2016). Importing agarwood-inducing fungal pathogens from other areas may pose potential environmental problems. Therefore, identifying locally available agarwood-inducing fungal pathogens from the specific region is essentially necessary.

Fungal identification can be carried out using morphological and molecular characterizations. Morphological identification compares characteristics of new unknown isolate morphologies to the reference species (Afzal et al. 2013). The *Fusarium* sp. from various places showed different colony colors and development types (Suryawan 2015). Moreover, molecular identification may be made by sequencing the internal transcribed spacer (ITS). The ITS sequencing has been widely utilized in fungal molecular characterization to the species level (Schoch et al. 2012) of *F. solani, F. oxysporum,* and *F. proliferatum* (Tan et al. 2021), and agarwood-inducing fungi from West Nusa Tenggara (Nugraheni et al. 2015). Available and accurate information about the appropriate identity of locally available fungi-inducing agarwood would improve agarwood production in certain regions, particularly in the North Aceh Regency, Aceh Province. The availability of local fungal isolates capable of inducing agarwood formation would improve local production to fulfill the agarwood market demand and export quota from these regions.

Agarwood production in Aceh Province depends on harvesting naturally occurring products in the local forests. Such conditions have significantly constrained sustainable agarwood supply and reduced agarwood production. On the other hand, Aceh Province is one of the largest producers of the best quality natural agarwood. The North Aceh province is also known for having the best fungal isolate capable of inducing natural agarwood. Since nature-dependent agarwood production is not sustainable, managing production through *Aquilaria* sp. cultivation and controlled inoculation of appropriate fungi to induce agarwood formation are necessary (Lukman et al. 2022). Controlled inoculation is carried out using locally selected isolates to reduce the potential adverse effects of introducing non-native fungal pathogens to the environment. Therefore, isolation and identification of local isolates of fungi capable of inducing agarwood formation from *Aquilaria* sp. is essential. This study aims to isolate local strains of fungi associated with agarwood formation from North Aceh regency, Aceh province, Indonesia, figure out their virulence against and ability to induce agarwood production from seedlings of *Aquilaria* sp., and determine their species identity based on the colony morphologies and ITS sequences.

MATERIALS AND METHODS

Isolation and identification of fungi

Naturally infected agarwood forming branches of *Aquilaria* sp. were collected from Teupin Resep Village, Sawang District, North Aceh Regency, Aceh Province, Indonesia. Fungal isolates and single colony isolation were conducted at the Forest Microbiology Laboratory, Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Indonesia. The inoculum sources used in the study were infected and agarwood forming branches of *Aquilaria* sp. The branches were cut into approximately 1×1×1 cm^3, dipped into 70% alcohol in a beaker glass for 10 sec to eliminate the surface contamination, rinsed four times by dipping them into sterile aquadest, and used as explants. The explants were planted onto P5 medium in a laminar airflow cabinet (LAFC) and incubated for seven days at room temperature (27-28°C). The observations were made daily until the seventh day, and the growing mycelium was re-isolated and cultured on potato dextrose agar (PDA) media to obtain a single mycelium culture. Subsequently, the pure fungal cultures were numbered as unique isolates. The isolated fungi were grouped based on their colony color and other morphological characteristics, such as the presence of macro-and microconidia, sporodochia, and chlamydospores. The fungal morphological identification was carried out macroscopically based on the procedure described by Alsohaili and Bani-Hasan (2018) and microscopically based on Lestari et al. (2021) methods. Microscopically identified fungal isolates were then proliferated in a potato dextrose broth (PDB) medium for further processing.

Virulence test

A total of five fungal isolates representing each previously identified isolate were tested for their virulence. Four evaluated isolates represent colony colors and types similar to the previously isolated fungi (GSL1 and GSL2 isolates, Faisal et al. 2020), and the other one represents distinct colony colors and types. The virulence test was conducted using 18-month-old seedlings of *Aquilaria* sp. in the glass house at the Leuwikopo Field Station, IPB. The virulence test was carried out using modified Faizal et al. (2017) methods. The test was conducted by injuring the stems of evaluated seedlings 10 cm above the ground and inoculating the wounded tissues with the appropriate fungal isolates. The tested treatments include physical injury without isolate inoculation (P0) as the control treatment, physical injury followed by inoculation with isolate GSL1 (P1 treatment), with GSL2 (P2), with LAI-1 (P3), with LAI-2 (P4), with LAI-3 (P5), with LAI-4 (P6), and with LAI-5 (P7). The inoculation was done by depositing the evaluated isolates onto the wounded stems using a

sterilized toothpick. Each of the inoculated seedlings was then sealed and labeled accordingly. Fungal isolates capable of inducing distinctive stem colors and fragrances were validated further using ITS sequencing.

The changes in the wood color surrounding the inoculated areas were measured 12 months after treatments. The bark surrounding the inoculated area was peeled off, and the areas showing infection were measured using graph paper and expressed in centimeter squares (cm^2). The presence of specific fragrances as early indicators of agarwood formation in the inoculated area was scored as either absent (0: no fragrance) or present (1: fragrance). All appropriate quantitative data were statistically analyzed using analysis of variance (ANOVA). Subsequently, mean comparisons were conducted using Duncan's multiple-range test (DMRT). All statistical analyses were performed using the R-Studio software.

Molecular characterization

Pure fungal isolates were cultured on a PDA medium, incubated at room temperature for ten days, and used as inoculum sources. The fungal mycelia grew on the PDA medium (0.25 cm^2), was cut and transferred onto the PDB medium, and cultures were shaken on a shaker at 125 rpm for 21 days. Subsequently, the growing mycelia of each fungal isolate on the PDB were harvested for DNA isolation. Total fungal DNA extraction was performed using the CTAB methods described by Legiastuti and Aminingsih (2012). Polyvinyl pyrrolidone (PVP) was freshly added to the CTAB extraction buffer, and sodium acetate precipitated the total DNA. Upon centrifugation, the DNA pellet was washed with 500 µL of 70% ethanol, air-dried, and resuspended with 100 µL of TE buffer. The isolated total DNA quality was tested visually using 0.8% agarose gel electrophoresis, and the quantity was measured using a spectrophotometer.

The ITS fragment PCR amplification was performed using the forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pairs. The PCR reaction (total volume of 25 µL) comprised of DNA sample (1 µL of 25-50 ng μL^{-1}), nuclease-free water (18.8 µL), 10x Taq Buffer with $(NH_4)_2SO4$ (ThermoFisher Scientifics, USA), dNTP (0.5 µL of 10 mM); each of the forward ITS1 and reverse ITS4 primers (1 µL of 10 µM), and the recombinant Taq DNA polymerase (0.2 µL of 5U μL^{-1}) (Dream Taq DNA Polymerase, ThermoFisher Scientifics, USA). PCR amplification was carried out by initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, primer extension at 72°C for 2 min, and followed by a final extension step at 72°C for 10 min. The amplified ITS was evaluated using agarose gel electrophoresis (1%) in Tris-Boric acid-EDTA (TBE, 0.5x) and visualized on a UV transilluminator (Sambrook and Russel 2012).

The PCR-amplified ITS fragment from each evaluated isolate was sent to a DNA sequencing service company (PT Indolab Utama Jakarta) for direct sequencing of the PCR products. After trimming the low-quality end, the determined nucleotide sequences were further evaluated using the basic local alignment search tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the fungal species. Moreover, the NCBI accessions identified from BLAST analysis were also retrieved and used for multiple sequence alignment (MSA) analysis using Geneious Prime version 2021.2.2 (https://www.geneious.com). Subsequently, the MSA output was used to construct a phylogenetic tree for the evaluated fungal isolates and the accessions retrieved from the NCBI database. The genetic distance was calculated using Tamura and Nei and the tree building using the Neighbor-Joining method using Geneious Prime version 2021.2.2 (https://www.geneious.com) with *Fusarium falciforme*, accession number MN326660.2 as the outgroup. Two previously reported fungal accessions from Indonesia (Faisal et al. 2020), capable of inducing agarwood from *Aquilaria* sp. and *Gyrinops* sp., and 22 accessions from the NCBI Genbank DNA Database were included as references in the analysis.

RESULTS AND DISCUSSION

Isolation and identification of fungi

An array of fungi growing from *Aquilaria* sp. cuttings were isolated. Macroscopically, the appearance of isolated fungal cultures is not easy to distinguish. However, comparing the fungal colony colors grouped the accessions into five types (Figures 1A-E). The LAI-1 colony color is white with a small quantity of purplish color (Figure 1A), LAI-2 is white (Figure 1B), LAI-3 is white with a quarter of purplish color (Figure 1C), LAI-4 is purple with thin mycelia sticking into the media (Figure 1D), and LAI-5 is white with a combination of slightly dark and thick mycelia (Figure 1E). Many thick mycelia appear at the top of the LAI-5 colonies. The LAI-5 also secretes substances that change the medium color to dark (1E).

All isolates met the criteria for fungi since the isolates form hyphae and insulate. The evaluated fungi's morphological characteristics include colony colors, macroconidia, microconidia, sporodochia, and chlamydospores. Only one out of five fungal isolates formed macro-and microconidia (Figures 1G-H), while the other four isolates (Figure 1F, Figure 1I, Figure 1J, Figure 1K) did not show any macroconidia at the time of observation. Budi et al. (2010) suggested that some fungal species did not produce sporodochia unless exposed to a particular light treatment during incubation.

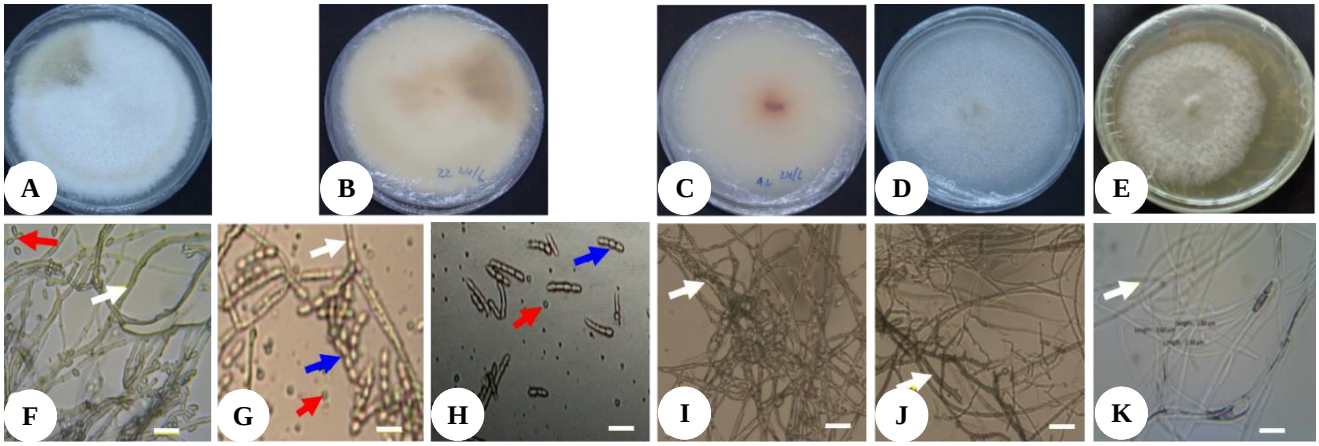

Figure 1. Pure cultures of agarwood-inducing fungi. A-E. Macroscopic observations. F-K: Microscopic observations (1.000x magnification). Red arrows indicate microconidia, yellow arrows indicate fungal hyphae, blue arrows indicate macroconidia, white bars indicate size references (10µm)

Based on the observed mycelium, LAI-1-4 (Figures 1A-D) could be *Fusarium* sp. because the fungal colony color is similar to *Fusarium* references (GSL1 and GSL2). The fungal isolates from North Aceh regency, Aceh province, showed various colony colors and morphologies. The colony color ranged from white to dark purple, straight to curved macroconidia, and contained 4-6 septas (Figure 1G-J), which were all *Fusarium* characteristics. *Fusarium* sp. produced microconidium and macroconidium, branching conidiophores, crescent-shaped macroconidium, trim stems, and often in pairs (Akhsan 1996). Meanwhile, the LAI-5 (Figure 1K) shows a different colony color than *Fusarium* references and might not be *Fusarium*.

Fusarium species identification could be made using several methods, such as macroconidia, microconidia, and chlamydospore colors (Hafizi et al. 2013). Budi et al. (2010) stated that the presence of conidia from sporodochia in fungal cultures is the main characteristic of *Fusarium* sp. Du et al. (2022) have asserted 16 genera of endophytic fungi capable of inducing agarwood production, most of which are from the *Fusarium* genera. In this study, no such conidium cell structure was observed. However, isolates produce elongated and insulated microconidia cell structure characteristics that were observed (Figure 1G). The absence of a cell structure resembling a crescent moon might suggest that the *Fusarium* species isolated from the North Aceh regency, Aceh Province, may differ from isolates from other areas. Different environmental conditions may also result in different *Fusarium*. Faizal et al. (2020) dan Mohammad et al. (2021) stated that the *Fusarium* has many species and different virulence levels.

Virulence tests

Five fungal isolates from North Aceh regency can induce color changes surrounding the infection areas. Moreover, the inoculated seedling stems with the fungal isolates from North Aceh regency also emit fragrance 12 months after inoculation, indicating agarwood formation. The discoloration areas (color changes) due to fungal infection and the scoring of induced fragrance 12 months after initial inoculation with the evaluated fungal isolates from North Aceh regency in the virulence test and fragrance scoring test were presented (Table 1).

Inoculation treatment with LAI-1-5 fungal isolates from North Aceh regency, Aceh Province, induced seedling stem discoloration, indicating the seedling's defense responses are activated because of the fungal inoculation. However, the areas of discoloration depended on the applied fungal isolates (Table 1). The stem discoloration tends to follow vertical paths of the plant vascular tissues. The stem discoloration changes are not just because of wounding but of inoculation treatments. The wounding in the control treatment did not result in stem discoloration responses like wounding and fungal inoculation (Figure 2A). The discoloration among inoculated seedlings started to occur nine months after inoculation, and the healthy white stems changed to brownies colors. In addition to stem discoloration, the inoculated seedling stem also changes its average score of fragrance level. The seedling stems inoculated with LAI-1-LAI-4 fungi induce fragrance formation from the infected areas of the stems.

One of the evaluated fungal isolates (i.e., LAI-5) causes massive damage to the stems, and the inoculated seedlings eventually die (Figure 2H). However, the dead seedling stems from LAI-5 inoculations, emitting a specific fragrance distinctive from agarwood products. No observation was conducted on whether the seedling death was because of either a severe virulence of LAI-5 or the LAI-5 isolate-secreted host-specific toxin. The LAI-5 is not recommended as an inducer in agarwood production. In addition to severe virulence, LAI-5 may also be dangerous to other potential plant hosts.

The virulence levels of LAI-1, LAI-2, LAI-3, and LAI-4 are the same as the GSL1 and GSL2 reference control isolates. Visual representatives of stem discoloration surrounding the inoculated site 12 months after initial inoculation in the virulence test are presented in Figure 2A-H. The color changes of the seedling stem from white to brown and a distinctive agarwood fragrance indicate that the inoculated stem tissues contain resin or agarwood. The resin formation from seedlings of *A. microcarpa* accumulates in the stem tissues, resulting in discoloration (Siburian et al. 2013).

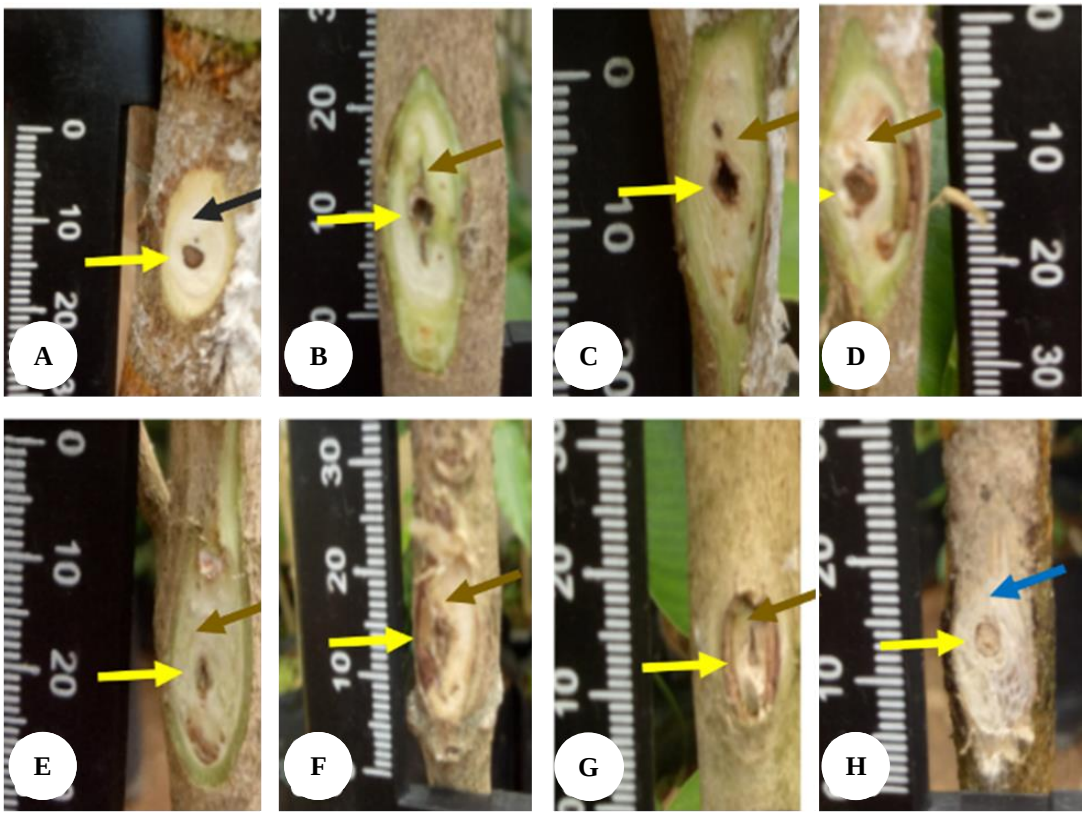

Figure 2. Visuals of color changes surrounding the inoculated site 12 months after initial inoculation with the evaluated fungal isolates in the virulence test. A. control, B. isolate GSL1, C. isolate GSL2, D. LAI-1, E. LAI-2, F. LAI-3, G. LAI-4, H. LAI-5. Yellow arrow: wounding and inoculation site positions, red arrow: induced agarwood formation areas, black arrow: healthy tissues that have not become agarwood, blue arrow: dead seedling stem

Table 1. The discoloration areas (color changes) surrounding the wounded areas after inoculation and induced fragrance level scores 12 months after initial inoculation with evaluated fungal isolates in the virulence test

Inoculation with and treatment codes	Discoloration areas (mm)	Fragrance level score
Control, wounding only (P0)	4.9^{d}	0.0^{c}
IsolateGSL1 (P1)	77.9^{a}	0.9^{a}
IsolateGSL2 (P2)	77.1^{ab}	0.9^{a}
LAI-1 (P3)	73.0^{bc}	0.7^{ab}
LAI-2 (P4)	70.7^{c}	0.7^{ab}
LAI-3 (P5)	71.5^{c}	0.6^{b}
LAI-4 (P6)	74.1^{abc}	0.9^{a}
LAI-5 (P7)	0.0$^{e\#}$	0.6$^{b\#}$

Note: Numbers followed by the same letter in the same column are not significantly different at α: 5%, according to the DMRT test. # The inoculated seedlings were not survived (dead)

The data showed that based on the discoloration level, the P1 treatment (inoculation with isolate GSL1) was better than others, followed by the P2 treatment (inoculation with GSL2) and P6 (with LAI-4) treatment (Table 1). Agarwood production using P2, P3, and P6 treatments induced the best fragrance score in the stem of 18-month-old seedlings of *Aquilaria* sp. Four tested isolates (LAI-1-LAI-4) induced agarwood formation, and one resulted in tested plants' death (LAI-5). The LAI-4 was the best isolate for inducing agarwood from *Aquilaria* sp. seedling stems compared to the other four fungal isolates (LAI-1, LAI-2, LAI-3 and LAI-5), and they induced the same response as isolate GSL1 and GSL2, as indicated by the fragrance score and the seedling stem discoloration surrounding the inoculation area. The LAI-1, 2, 3, and 4 fungal pathogens from North Aceh induced agarwood formation. Previous reports showed that inoculating appropriate fungal isolates would induce good-quality agarwood formation. Many fungal pure-culture isolated from natural agarwood are effective inducers for agarwood formation from *Aquilaria* sp trees (Cui et al. 2013; Sangareswari Nagajothi et al. 2016).

In this study, Indonesian isolates GSL1 and GSL2 and LAI-4 are the best fungal isolates for inducing agarwood, and LAI-1, LAI-2, LAI-3, and LAI-5 are not as good inducers as the other three isolates. Indonesian isolates GSL1 and GSL2, and LAI-4 induced discoloration and fragrance from seedling stems, indicating agarwood formation. Previous studies have suggested that agarwood stored in wood fibers resulted from volatile compound accumulation in the injured areas of the trees (Subasinghe and Hettiarachchi 2013; Jong et al. 2014). Agarwood formation is generally in small quantities, indicated by the

dicoloration in the surrounding injured or fungal-infected areas and the emission of a distinctive aroma when the agarwood is burned. The agarwood formation stops if the infected tree is resistant and continues if the tree is susceptible to the fungal inducers. Fungal-infected *Aquilaria* sp trees have developed self-defense mechanisms to limit fungal infection activities (Cui et al. 2013; Rasool and Mohamed 2016). A collection of fungal isolates capable of inducing agarwood are necessary. In nature, agarwood formation takes a long time, exceeding one to five years. Therefore, using fungal isolates to induce agarwood formation purposely will be beneficial. Moreover, sustainable agarwood and high-quality sapwood production should be possible with practical and controlled techniques.

Molecular characterization

Putative ITS fragments were successfully amplified using the ITS-specific primers. Examples of the PCR-amplified putative ITS from the four tested fungal isolates are presented. PCR amplification of ITS from LAI-1-4 fungi resulted in a single amplicon of approximately 510 bp. On the other hand, PCR amplification of ITS from Indonesian reference isolates GSL1 and GSL2 resulted in a single amplicon of approximately 1100 bp (Figure 3). Direct PCR product sequencing of the putative ITS fragments resulted in sequence lengths of 567 bp (LAI-1), 573 bp (LAI-4), 586 bp (LAI-3), 588 bp (LAI-2), 642 bp (GSL1isolate), and 1074 bp (GSL2). The determined ITS sequences were deposited in the NCBI DNA database under the following accession numbers OQ061205 (LAI-1), OQ061204(LAI-2), OQ061203 (LAI-3), OQ061202 (LAI-4), OQ061201 (LAI-5-1R), OQ061200 (LAI-5-1F), OQ061198 (LAI-5-3R), and OQ061199 (LAI-5-3F).

All putative ITS sequences were subjected to the basic local alignment search tool (BLAST) analysis against nucleotide accessions in the NCBI DNA database to confirm the identity of ITS fragments. Summary of the BLAST analysis results using query sequences of ITS from the evaluated LAI-1-LAI-4 from North Aceh regency, Indonesia, and reference GSL1 and GSL2 isolates are presented (Table 2). The output of BLAST analysis was used to determine the species identity of the evaluated fungal isolates based on their sequence similarity against reference ITS sequences in the NCBI DNA Database (Table 2).

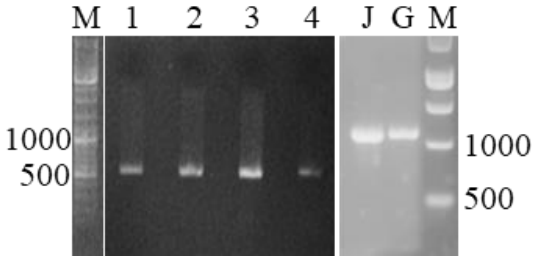

Figure 3. Gel photographs of PCR amplified ITS fragment generated using universal ITS-specific primers and total genomic DNA of fungal isolates capable of inducing agarwood. Columns 1-4: LAI-1-4, Column J: GSL2 isolate, column G: GSL1 isolate. M-represents the DNA size markers (100 bp DNA ladder). The indicated sizes are in base pairs

Table 2. Summary of basic local alignment search tool (BLAST) results using query sequences of ITS from the evaluated LAI-1-4 from North Aceh regency, Indonesia, and reference GSL1 and GSL2 isolates

Query sequences of ITS and species identity	NCBI accessions	Identity (%)	Query sequences of ITS and species identity	NCBI accessions	Identity (%)
LAI-1 (OQ061205):			**GSL1 isolate:**		
Fusarium solani	MK968891.1	99.5	*F. solani*	ON614833.1	99.8
F. solani	MF495401.1	99.5	*Fusarium* sp.	OP450953.1	99.8
F. solani	MH864902.1	99.5	*F. solani*	KU238143.1	99.6
F. solani	ON795988.1	99.5	*F. solani*	KU377470.1	99.4
F. solani	OM533682.1	99.5	*F. solani*	KF679356.1	99.4
LAI-2 (OQ061204):			**GSL2 isolate:**		
F. solani	KT336512.1	99.8	*F. solani*	KY245947.1	100
F. solani	LC002783.1	99.8	*F. solani*	MF281192.1	100
F. solani	OL719293.1	99.0	*F. falciforme*	MN326660.2	99.8
F. solani	MF687193.1	99.3	*F. bataticola*	AF178407.1	99.8
F. solani	ON795988.1	98.8	*F. haematococcum*	HQ696788.1	99.8
LAI-3 (OQ061203):			**LAI-5 isolate-Colony #1:**		
F. solani	MF495401.1	99.8	*Irpex laceratus*	MH734799.1	99.8
F. solani	ON795988.1	99.8	*I. laceratus*	KT844687.1	99.8
F. solani	OM533682.1	99.8	*Ceriporia lacerata*	KF782841.1	99.8
F. solani	0M533653.1	99.8	*I. laceratus*	MT912577.1	99.8
F. solani	OL410542	99.8	*C. lacerata*	KP326576.1	99.8
LAI-4 (OQ061202):			**LAI-5 isolate-Colony #3:**		
F. solani	MK968891.1	100	*Daldinia eschscholtzii*	ON426842.1	99.3
F. solani	MF495401.1	99.8	*D. eschscholtzii*	MN341731.1	99.5
F. solani	ON795988.11	99.8	*D. eschscholtzii*	MN368169.1	99.5
F. solani	OM533682.1	99.8	*D. eschscholtzii*	KY792621.1	99.3
F. solani	OM533653.1	99.8	*Daldinia* sp.	OP445264.1	99.1

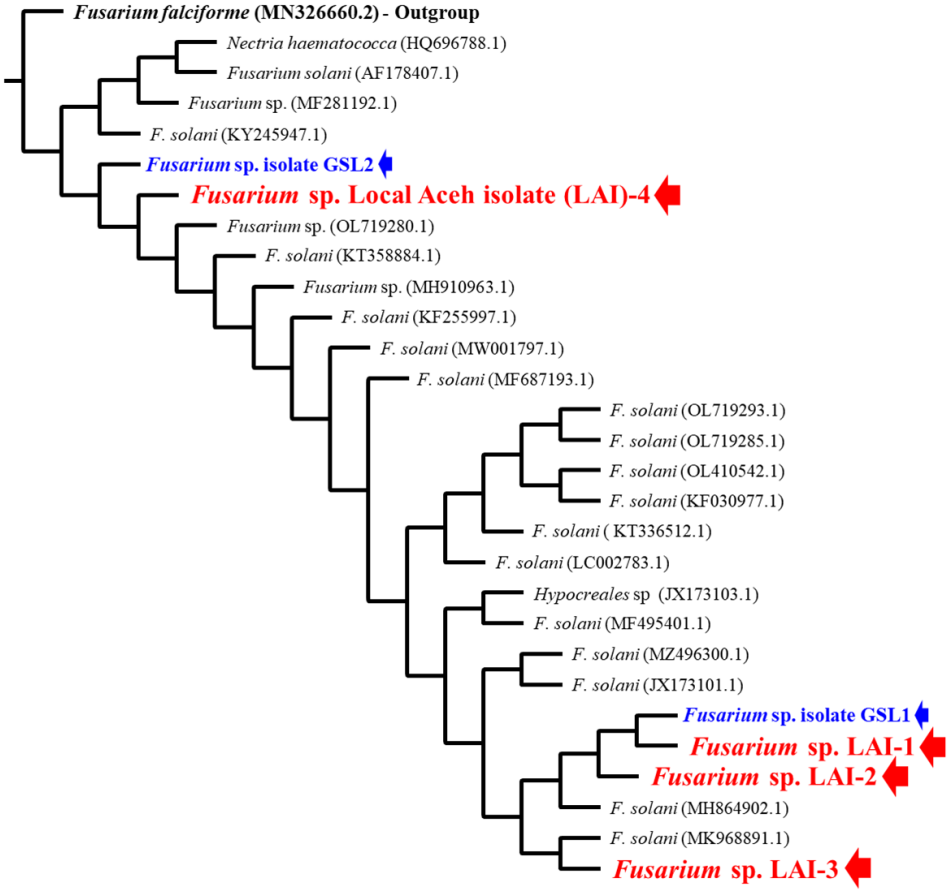

Figure 4. Phylogenetic tree based on ITS sequences of four fungal isolates (red arrow) from North Aceh regency capable of inducing the agarwood formation from seedlings of *Aquilaria* sp. The genetic distance was calculated using Tamura and Nei and the tree building using the Neighbor-Joining method with *Fusarium falciforme* (Ff), accession number MN326660.2, as the outgroup. Two previously reported fungal accessions (blue arrows) from Indonesia (Faisal et al. 2020), capable of inducing agarwood, and 22 accessions from the NCBI DNA Database were included as references in the analysis. The ITS sequences for *Fusarium* sp. Local Aceh isolate (LAI)-1-LAI-4 were deposited in the NCBI DNA database, accession numbers OQ061205, OQ061204, OQ061203, and OQ061202

The BLAST analysis showed that the putative ITS sequences are indeed ITS fragments. Based on the determined ITS nucleotide sequences, the LAI-1-LAI-4 and the reference Indonesian isolate GSL1 are members of *F. solani* (Table 2). The percentage identity of determined ITS sequences of LAI-1-LAI-4 and the reference Indonesian isolate GSL2 were more than 99% similar to *F. solani*. While the percentage identity of the reference Indonesian isolate GSL2 ITS sequences is more than 99% similar to either *F. solani*, *F. haematococcum*, *F. falciforme*, or *F. bataticola* (Table 2).

The BLAST analysis using ITS sequences of two LAI-5 colonies was also done to confirm the identity of the fungal isolate showing different morphology than *Fusarium* sp. and causing seedling death upon inoculation treatment. The BLAST analysis results using ITS sequences indicated that one of the two samples of LAI-5-colony #1 is either *Ceriporia lacerata* or *Irpex laceratus*, and the other of LAI-5-colony #3 is *Daldinia eschscholtzii* (Table 2). Based on these findings, the LA-5 isolate was probably a mixture of the two white rot fungus isolates.

The *C. lacerata* or *I. laceratus*, and *D. eschscholtzii* are wood-decaying fungi (Sahu et al. 2021), which was in line with the results of the fungal virulence test (Figure 2H). The ITS sequences could be used to determine the identity of fungal isolates at the species level. However, it is not a good indicator of the taxonomic order lower than the species. Therefore, the isolated fungi capable of inducing agarwood formation from North Aceh regency, Aceh province, are *F. solani*. Based on their ITS sequences, the LAI-1, LAI-2, and LAI-3 are closely related to the Indonesian reference isolate GSL1, while the LAI-4 is closely related to the Indonesian reference isolate GSL2 (Figure 4).

Daniels et al. (2013) also mention that the sequence of nucleotide bases is said to be identical if it has a minimum query coverage value of more than 40%. It can be assumed that the agarwood-inducing fungi isolated from North Aceh regency, Aceh Province, are the same *Fusarium* sp. as the previously reported reference Indonesian isolates GSL1 and GSL2. Although the same *Fusarium* sp. LAI-1-4 might be more suitable for inducing agarwood in North Aceh regency, Aceh Province, since they have the same

virulence levels as GSL1 and GSL2, and they are local isolates that would not pose potential problems to other crops in the regions. Wounding without fungal inoculation proved unable to induce agarwood formation. On the other hand, wounding and inoculation treatment with LAI-1, LAI-2, LAI-3, and LAI-4 effectively induced agarwood formation, and the induction was similar to the reference fungal isolates GSL1 and GSL2. The LAI-5 is a different species than LAI-1-LAI-4 and is unsuitable for agarwood induction since the isolate could kill the inoculated plants.

Based on the identification results, the fungi capable of inducing agarwood formation are *F. solani* which are pathogenic to *Aquilaria* sp. Until now, there is no report stating that fungal inoculation to agarwood-producing plants causes damage to the environment. Nonetheless, *F. solani* was reported as pathogenic to various plants (Rita et al. 2013; Abedi-Tizaki et al. 2016; Ganesh and Dwivedi 2019; Lestari et al. 2021; Poss et al. 2022). Therefore, specific care needs to be taken before releasing introduced fungi to North Aceh.

On the other hand, the pathogenicity of *F. solani* can still be controlled by certain plants (Poss et al. 2022) that are non-hosts of the fungi. Because agarwood production takes up to eight years, it is recommended to cultivate *Aquilaria* sp. using an intercropping system. The recommended intercropping crops should be other than *Solanaceae* species since this species will become an alternative host to the fungi. Some recommended crops include mango, coconut, rambutan, and areca nut. Therefore, the farmers could use the available gardens to do intercropping among *Aquilaria* sp. with such commercially valuable trees.

In conclusion, four fungal isolates were successfully obtained using agarwood of *Aquilaria* sp. from North Aceh. The pure isolates derived from single mycelium culture (LAI-1, LAI-2, LAI-3 and LAI-4) were pathogenic to *Aquilaria* sp. seedlings, indicating they can induce agarwood formation. ITS sequences of the LAI-1, LAI-2, LAI-3 and LAI-4 indicate that they are all *Fusarium* sp. Since the LAI-1, LAI-2, LAI-3 and LAI-4 are all local isolates from North Aceh, local farmers could use them to induce agarwood formation from *Aquilaria* sp. purposely. Therefore, cultivation of *Aquilaria* sp. and induction using identified local isolates may be used as alternatives in sustainable agarwood production in the areas in the future. Developing intercropping among *Aquilaria* sp. and other commercially valuable trees are suggested to minimize the possible risk of environmental effects and develop sustainable agarwood production in the future. Further research on the intercropping of *Aquilaria* sp. and other trees should be the subject of future investigations.

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