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Agarwood Formation Induced by Fermentation Liquid of *Lasiodiplodia theobromae*, the Dominating Fungus in Wounded Wood of *Aquilaria sinensis*

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Abstract Agarwood is broadly used in incense and medicine. Traditionally, agarwood formation is induced by wounding the trunks and branches of some species of *Aquilaria* spp., including *A. sinensis*. As recently evidenced, some fungi or their fermentation liquid may have the potential of inducing agarwood formation. The present study aimed to analyze the fungi isolated from an agarwood-producing *A. sinensis* tree and subsequently identify the fungi capable of promoting agarwood formation. We identified a total of 110 fungi isolates based on their morphological characteristics and rDNA ITS sequences. These isolates came from four different layers (namely the decomposing layer, agarwood layer, transition layer, and normal layer) near the agarwood formation site of the trunk. According to the experimental results, most of them belonged to *Dothideomycetes* (81.82%), while the others to *Sordariomycetes* (13.64%) or *Eurotiomycetes* (4.55%). Of note, 88 isolates were shown belonging to the species of *Lasiodiplodia theobromae* that are most frequently isolated from different layers. In addition, when the fermentation liquid of two isolates of *L. theobromae* (AF4 and AF12) and one isolate of *Fusarium solani* (AF21) was inoculated into the *A. sinensis* wood using the Agar-Wit technique, promoted agarwood formation was observed; however, the effect of AF21 did not keep stable in the later test, while AF4 and AF12 still functioned 1 year later. This study may lay a foundation for exploring the underlying mechanism of agarwood formation as well as fungi application in agarwood production.

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Introduction

Agarwood is widely used in perfumery, traditional medicine and incense in ceremony [8, 9]. Actually, it is the resinous portion of the trunk and branches of *Aquilaria, Gonystulus*, and *Gyrinops* species (Thymelaeaceae) [16, 18, 48], and cannot be produced in a healthy tree under a natural environment. Agarwood formation is only available under certain external factors, such as lightning strike, animal grazing, insect attack, or microbial invasion, typically around wounded or rotting parts of the trunk [3, 31, 36]. Because of the rareness and slow formation of wild agarwood, some artificial agarwood-inducing methods have been developed, especially for the endangered agarwood-producing species. Most previous artificial techniques focused on imitating natural agarwood formation using axes, knives and nails to wound tree trunks and branches [30]. Noticing the fungi infection that usually accompanies the physical wounding of trees, some researchers showed interest in promoting agarwood formation via fungi. The fungi-inoculation method was first presented by Tunstall in 1929 [13], and then introduced into China in 1976. The research concerning *Aquilaria* callus has showed that some plant elicitors, such as jasmonic acid and methyl jasmonate, are capable of inducing the production of representative chemical components in agarwood [19, 21, 33]. Our laboratory has developed a significant method—the whole-tree agarwood-inducing technique (Agar-Wit) [43]. By this method, an agarwood inducer is inoculated into the xylem

part of *Aquilaria* trees through cheap and simple transfusion sets. Along with water transportation, the inducer is transported throughout a tree, and consequently induces an overall wound, resulting in agarwood formation in a short period of time [24, 44].

The opinions regarding the mechanism of agarwood formation generally fall into two categories: (1) agarwood forms when a tree is physically wounded and simultaneously infected by fungi [28, 40]; (2) agarwood forms as long as a tree is physically wounded, regardless of fungi infection [29, 36]. According to our recent research, a wounding treatment enables the biosynthesis of sesquiterpene (a representative agarwood substance) as well as the formation of the typical structure vessel occlusion in the stems of *A. sinensis* trees, without causing any variation of microbial communities [51]. Under the hypothesis presented in [52], agarwood is a response of plant defense [52]. When an *Aquilaria* tree is wounded, damage signals are induced and transmitted to activate the defense response, which subsequently leads to the production of defensive substances such as sesquiterpenes and phenylethyl chromone derivatives. These secondary metabolites are imbedded in the wood tissue to avoid damage expansion, resulting in agarwood formation [46]. The causal wounding agents include physical, chemical, and fungi-infecting and elicitors-inducing factors.

Different methods for tree wounding result in different qualities of agarwood [49]. Among these methods, physical wounding methods usually harvest high-quality agarwood, but will take a few years or even one decade. The Agar-Wit technique developed in our lab realizes a much faster agarwood formation while causing less damage to the endangered natural resources [24, 43, 44]. We are now making efforts to increase the agarwood quality and further shorten the time required for artificial agarwood production by this technique. It has been commonly accepted that some fungi have potential in accelerating agarwood formation and improving agarwood quality. In 1976, the researcher from Guangdong Institute of Botany found that fungi infection of *A. sinensis* led to agarwood formation. Qi et al. [34] reported that *Menanotus flavolives* infection of *A. sinensis* accelerated agarwood formation. Gibson [13] isolated an endophytic *Cytosphaera mangiferae*, which led to agarwood formation when inoculated to healthy trees. Subeham et al. [39] inoculated *Fusarium laseritum* into the holes on the trunk of *Aquilaria* sp. trees and obtained agarwood one year later. Feng [12] isolated nine strains from the agarwood of *A. sinensis*, and identified four species (*Botryosphaeria rhodina, Hypocrea jecorina, Trichoderma reesei, T. koningii*) capable of promoting the formation of good-quality agarwood. Xu [45] evidenced that *Fusarium* sp. promoted agarwood formation one year after fungi inoculation.

In this work, an agarwood-forming site in a tree of *A. sinensis* was dissected into four layers, including the decomposing layer (DL), agarwood layer (AL), transition layer (TL), and normal layer (NL) [15] from which fungi were respectively isolated and identified. Using Agar-Wit technique, the fermentation liquid of thirteen isolates was screened and evidenced to play a certain role in stimulating agarwood formation.

Materials and Methods

Plant Materials

From an 8-year-old tree grown in the *A. sinensis* plantation garden of Hainan Branch Institute of Medicinal Plant Development, located in Hainan Province of China, was cut off a branch sprouting from the main truck. The cutting site was about one meter above the ground, with the section diameter of about eight centimeters (Fig. 1a). Two years later, when agarwood formed, woody samples were collected, respectively, from the DL, AL, TL, and NL near the agarwood formation site (Fig. 1b), and then cut into 3×3 cm blocks for further layer cutting.

Fungi Isolation

Surface sterilization was performed according to Dobranic et al. with some modification [10]. The woody blocks were first washed thoroughly in running tap water, then immerged successively in 70% ethanol for 3–5 min and 10% sodium hypochlorite for 5 min, and finally rinsed in sterile distilled water for three times. Although layer distinction was somewhat difficult because of the thin-layered AL, a dark line usually appeared when agarwood formed. Then fifteen sterile blocks of 3×3 mm each were randomly cut using a cork borer for each layer, and placed in 90-mm-diameter petri dishes at three blocks per dish, which contained potato dextrose agar (PDA) medium (potato 200 g, dextrose 20 g and agar 15 g in 1 L) with ampicillin 100 mg/L to suppress bacterium contamination. Different layers were found to have different cutting characteristics. The DL was soft, loose, and easy to cut, the AL was hard and difficult to cut, and the TL had hardness between the AL and NL. To avoid cross-contamination between different layers, both the cork borer and the cut slices were sterilized. Next, all the petri dishes were sealed by parafilm 'M' and incubated in a light chamber at 12-h light/dark cycles at 28 ± 2 °C. Five days later, new fungal colonies were monitored or picked out everyday, and this process lasted at least 2 weeks until all the fungi were separated as single colonies. Individual fungal colonies were

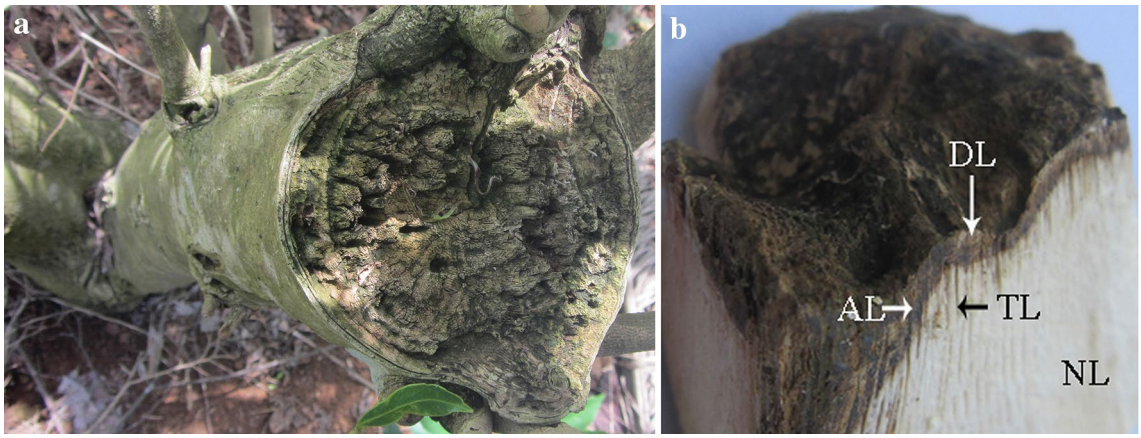

Fig. 1 Agarwood induced by the branch-cutting method was used for fungi isolation in the present study. Four layers were separately collected. *DL* decomposing layer, *AL* agarwood layer, *TL* transition layer, *NL* normal layer

picked from the edge with a sterile fine-tipped needle and transferred onto PDA. After subculture, these fungi were stored at the Hainan Branch Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. AF4 and AF12 (CGMCC No.9595) were deposited as living cultures in China General Microbiological Culture Collection Center.

ITS Sequences Analysis

After subculture of fungal isolates grown on PDA for 5 days, fresh mycelia were inoculated into a 100 mL Erlenmeyer flask containing 50 mL liquid potato dextrose (PD) medium and cultured in a shaking incubator at 120 rpm/min for 3–15 days in darkness at 28 °C. Mycelia from each fungus were obtained by vacuum filtration. The total genomic fungal DNA was extracted by the plant genomic DNA kit (TIANGEN, CHINA). Each fungal DNA was amplified with primers ITS1 (5′-TCCGATGGTGAACCT GCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′). The amplification was performed in a 25 μL reaction volume containing 100 ng template DNA, 1 μL of 10 pmol of each primer, and 12.5 μL of 2× PCR Master-Mix (TIANGEN, CHINA). The thermal cycling program was as follows: 5 min initial denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 50 °C, 1 min extension at 72 °C, and a final 7 min extension at 72 °C. A negative control using water instead of template DNA was included in the amplification process. From each PCR reaction, 5 μL of PCR products was examined by agarose gel electrophoresis at 0.8% (w/v) using ethidiumbromide staining. PCR products with distinct bands were sequenced using the primer pairs ITS1 and ITS4 on an ABI 3730 XL sequencer (SANGON, CHINA). All the fungal sequences were blasted in GenBank.

GenBank accession Number and the closely related species from GenBank.

Four layers	Strains	Genbank accession No.	Closely related species (GenBank accession No. and similarity %)
DL layers	DL1	KX650826	*Asperqillus niger* (Gu951769 and Ku882054, 99%)
	DL2	KX650827	*Nigrospora* sp. (KT192335 and KU504328, 99%)
	DL3	KX650828	*Lasiodiplodia theobromae* (JX868613 and KJ381073, 100%)
	DL4	KX650829	*Penicillium purpurogenum* (HQ907949 and HQ637358, 99%)
	DL5	KX650830	*Lasiodiplodia pseudotheobromae* (AB873041 and KT208383, 99%)
AL layers	AL1	KX650831	*Fusarium solani* (HQ384397, 99% and KF494130, 100%)
	AL2	KX650832	*Hypocrea lixii* (JN704349 and FJ461561, 99%)
	AF3	KX650833	*Lasiodiplodia theobromae* (KM357551,100% and KM357551, 99%)
	AL4	KX650834	*Megacapitula villosa* (KC771508 and JN128868, 99%)
	AL5	KX650835	*Purpureocillium lilacinum* (KT310927 and JN851054, 99)

Four layers	Strains	Genbank accession No.	Closely related species (GenBank accession No. and similarity %)
	AL6	KX650836	*Fusarium* sp. (EU707572 and HM535403, 99%)
	AF7	KX650837	*Lasiodiplodia theobromae* (KM357551 and KJ612075, 99%)
TL layers	TL1	KX650838	*Lasiodiplodia theobromae* (KT211557 and KR260793, 100%)
	TL2	KX650839	*Purpureocillium lilacinum* (LT220738 and KT968534, 100%)
NL layers	NL1	KX650840	*Fusarium* sp. (EU707572 and HM535403, 99%)
	NL2	KX650841	*spergillus wentii* (HM014129 and EF652151, 99%)
	NL3	KX650842	*Lasiodiplodia theobromae* (HM466951 and FJ612656, 99%)
	NL21	KX650843	*Fusarium solani* (KJ009328 and KF751072, 99%)
	NL5	KX650844	*Aspergillus oryzae* (KM999948 and HQ285580, 99%)

For some strains showed the same morphological characteristics and ITS sequence in the four layers, only one strain as representative was submitted to GenBank.

Further Verification of Agarwood-Inducing Fungus by EF-Alpha Gene

To verify identification results obtained by ITS sequence alignment, primers EF1T (5′-ATGGGTAAGGAGGAC AAGAC-3′) and EF2T (5′-GGAAGTACCAGTGATCAT GTT-3′) were used to amplify EF-alpha gene. The reaction was performed in a 25 μL total volume containing 100 ng template DNA, 1 μL of 10 pmol of each primer, and 12.5 μL of 2×PCR MasterMix (TIANGEN, CHINA). The thermal cycling program was: initial denaturation at 94 °C for 85 s; 35 cycles of denaturation at 95 °C for 35 s, annealing at 57 °C for 55 s, extension at 72 °C for 1 min; and a final 10 min extension at 75 °C. The EF-alpha gene sequence of isolate AF21 was submitted to GenBank (No. KY091148) and was aligned to be the sequence of *Fusarium solani* which is the same as the ITS identification. Therefore, the result analysis was conducted based on the ITS identification as well as the morphological characters.

Morphological Characterization

The isolates were inoculated on PDA plates and grow in an incubator at 28 C for 7 days. Morphological observation was carried out when the mycelium of each isolate occupied the whole Petri plate. Morphological characteristics included macroscopic and microscopic characteristics. Macroscopic characteristics included the colony morphology and growth rate of the fungal isolates and microscopic characteristics included the conidial shape and size and color. If the fungi cannot produce spore in 7 days, we had to induce spore production by light culture for a lone time. The morphology of the fungi was observed on the slides and coverslips by scanning electron microscope (OLYMPUS SZX16) at Hainan Academy of Agricultural Sciences.

Screening of Agarwood-Promoting Fungi

All together thirteen isolates (one isolate each from the species of *Megacapitula villosa, Lasiodiplodia pseudotheobromae, Nigrospora* sp., *Fusarium* sp., *Fusarium solani, Trichoderma harzianum, Paecilomyces lilacinus, Penicillium purpurogenum, Aspergillus wentii, Aspergillus oryzae, Aspergillus niger* and two isolates from *Lasiodiplodia theobromae*) were cultured in the liquid PDA medium for 7 days. The two isolates of *L. theobromae* showed distinct growth characteristics, in different colors and shapes. The mycelium was filtered, and then the remaining fermentation liquid was inoculated into the wood of 3-year-old *A. sinensis* trees using the Agar–Wit technique [43]. The trees were planted in the *A. sinensis* plantation garden of Hainan Branch Institute of Medicinal Plant Development located in Hainan Province of China. A hole of 5 cm in diameter was made in the stems 50 cm above the ground. A total of 200 mL fermentation liquid was inoculated for each tree, and three trees were used for each treatment. The patented agarwood inducer-[44] and culture medium-inoculated trees were taken as control. Two months later, the woods were cut at 10 cm above and below the inoculated sites (IW) (at the trunk 30 cm above the ground). Agarwood-like materials from the inoculated trees were separately collected, and all fresh samples were naturally dried in shade. Whether agarwood had been formed was determined by the chromone content in the wood using the thin-layer chromatography (TLC) method. Because sesquiterpenoids and 7-dimethoxy-2-(2-ph enylethyl) chromone are the well-known characteristic active compounds of agarwood, and no chromone compounds exist in the healthy wood of *A. sinensis*, we chose the chromone as the detection index.

The TLC was conducted as follows: First, the wood or agarwood was crushed by a pulverizer and filtered with 26 meshes. Then, 1 g powder was extracted in 25 mL methanol for 30 min by the ultrasonic method (Ultrasonic Cleaner, 59 kHz, 500 W, SK8200H, China). The extract was filtered and evaporated to dryness under 80 °C water baths. The residue was dissolved in methanol and adjusted to 5 mL Chromone (isolated previously in our laboratory) and was used as standard constituents (ST). Finally, 2 μL solvent was drawn into a capillary, and then pressed onto the TLC plate (GF_{254}, 10 × 20 cm, Qingdao Haiyang Chemical Co., Ltd., China). The mobile phase used $CHCl_3$:Et_2O (10:1, v/v). The TLC plate was developed and then visualized under UV_{254}.

Results

Fungi Isolation and General Characterization

A total of 110 fungi isolates were obtained in the present study. Based on morphological characteristics and rDNA ITS sequences, 90 isolates (81.82%) were determined to be members of *Dothideomycetes*, 15 isolates (13.64%) members of *Sordariomycetes*, and 5 isolates (4.55%) members of *Eurotiomycetes*. In the class of *Dothideomycetes*, only one isolate belonged to the order of *Pleosporales*, while all the other 89 isolates belonged to the order of *Botryosphaeriales*. In the class of *Sordariomycetes*, one isolate belonged to the order of *Trichosphaeriales*, while the other 14 isolates belonged to the order of *Hypocreales*. All the five isolates of *Eurotiomycetes* belonged to the order of *Eurotiales*. Among all isolates, 106 isolates were identified at the species level, and only four isolates could not be classified into any given species, of which one belonged to the family of *Nigrospora*, and the other three to the family of *Fusarium*. Taken together, ten species and three unidentified species were isolated in the present study. It is worth noting that 88 isolates belonged to the species of *L. theobromae*.

Characterization of Fungi Spreading in Different Wood Layers of Agarwood Formation

To explore the fungal function in agarwood formation, four woody layers were defined. Thirty-seven (33.64%) fungi were isolated from the DL, 46 (41.82%) from the AL, 18 (16.36%) from the TL, and 9 (8.18%) from the NL. As shown in Fig. 2, *L. theobromae* was the dominant species in all the four layers. *Fusarium solani* was isolated from both the AL and NL, respectively, with the number of two and one. *Paecilomyces lilacinus* was isolated from both the AL and TL, respectively.

Identification of Fungi Capable of Promoting Agarwood Formation

To identify new fungi capable of promoting agarwood formation, the fermentation liquid of thirteen isolates which showed different morphological characteristics was tested using the Agar–Wit technique. The results show that the fermentation liquid of three isolates (two of *L. theobromae*, termed as AF4 and AF12, and one of *F. solani*, termed as AF21) had potential in promoting agarwood formation (Fig. 3a). One month after the inoculation of fermentation liquid of AF4, AF12, and AF21 into the wood of *A. sinensis*, the leaves gradually turned yellow (Fig. 3b); two months later, the wood turned brown, just like the trees inoculated with the patented agarwood inducer (Fig. 3a). While in the culture medium and the NAF6 fermentation liquid-inoculation groups, the wood of trees remained unchanged. Noticeably, all the fermentation liquid of AF4, AF12, and AF21, as well as the patented agarwood inducer, played a positive role in promoting agarwood formation; AF4 and AF12 outperformed AF12. Next, the extracts were analyzed by TLC to test if characteristic agarwood substances had been formed. The 6,7-dimethoxy-2-(2-phenylethyl) chromone, a standard component, was determined to have an R_f value of 0.65. All the trees inoculated, respectively, with the fermentation liquid of AF4, AF12, and AF21, as well as the patented agarwood inducer, developed bright blue spots in the same position as standard chromone, while the culture medium-inoculated wood showed no spot on the TLC plate (Fig. 3g). We therefore draw the conclusion that the fermentation liquid of two isolates of *L. theobromae* (termed as AF4 and AF12) and one isolate of *F. solani* (termed as AF21) can induce *Aquilaria* trees to form agarwood through the Agar-Wit technique. The colonies of AF4, AF12, AF21, and NAF6 are shown in Fig. 3c, d, e, f, respectively. Although *Fusarium solani* (AF21) could promote the agarwood formation at the initial stage, its effect was not stable in the later test, while *L. theobromae* (AF4 and AF12) was still capable of promoting agarwood formation 1 year later.

Discussion

The identification and isolation of fungi from the healthy tissues or agarwood of *A. sinensis* and other agarwood-producing species have been extensively reported. Gong et al. [14] isolated 128 fungi from the leaves, roots, and stems of *A. sinensis*, with *Fusirium* sp. 1 and *Glomerularia* sp. as the

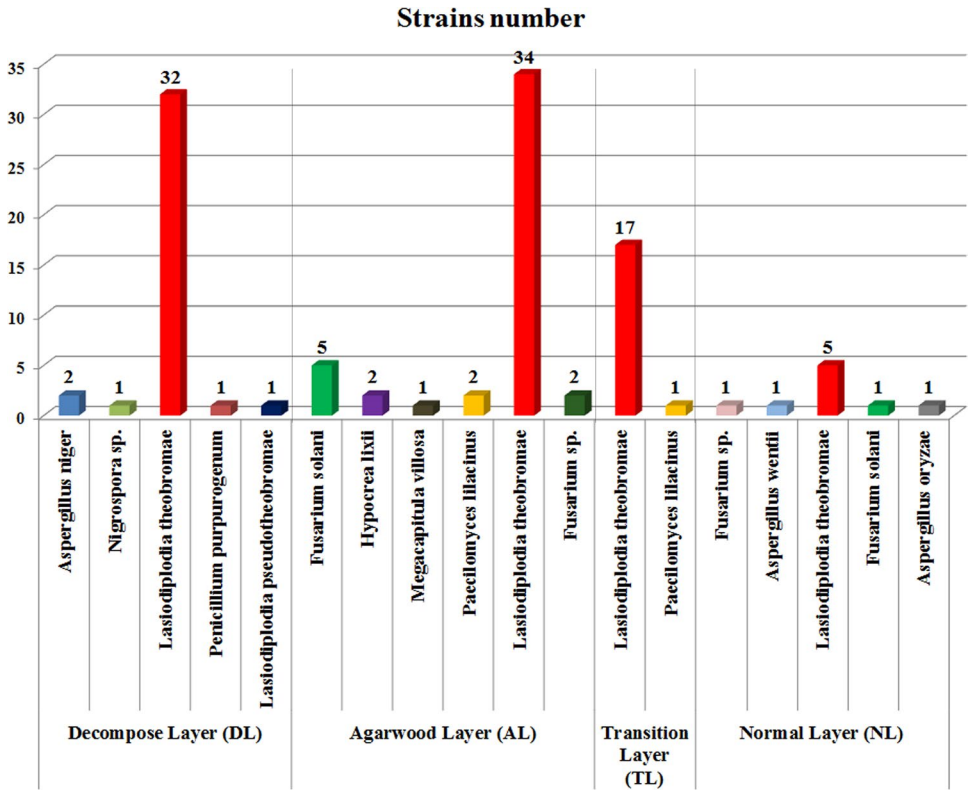

Fig. 2 The numbers of isolated strains from different layers. The species of *Lasiodiplodia theobromae* was dominantly spreading in each layer

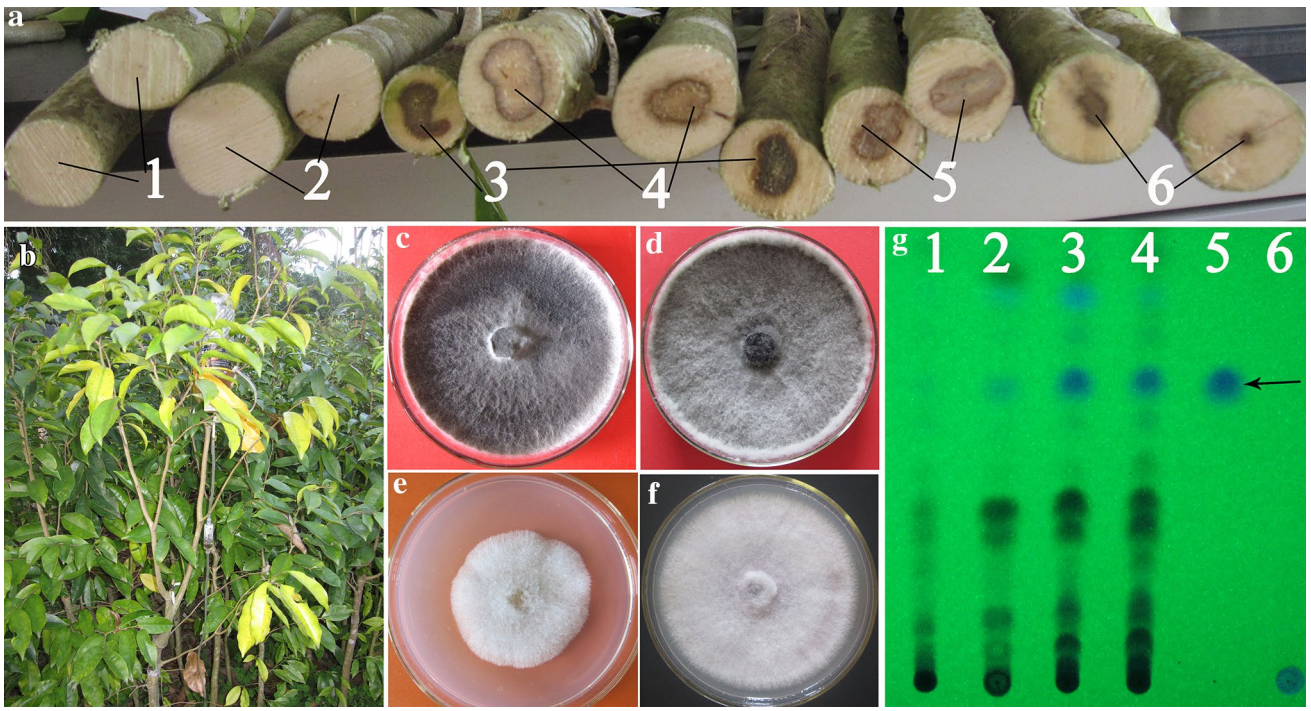

Fig. 3 Agarwood induced by the fermentation liquid of isolated fungi. **a** Wood inoculated with: *1* the fermentation liquid of NAF6; *2* culture media; *3* the fermentation liquid of AF12; *4* the fermentation liquid of AF4; *5* the patented agarwood inducer; *6* the fermentation liquid of AF21. **b** Plants that were inoculated with the fermentation liquid of fungi showed *yellow* leaves; **c** colony of AF4; **d** colony of AF12; **e** colony of NAF6; **f** colony of AF21; **g** thin-layer chromatogram of extracts from the wood inoculated with: *1* the fermentation liquid of AF21; *2* the patented agarwood inducer; *3* the fermentation liquid of AF4; *4* the fermentation liquid of AF12; *5* ST; *6* 7-dimethoxy-2-(2-phenylethyl) chromone, and six culture media. (Color figure online)

dominant species. Wang et al. [42] isolated 50 fungi from the stems, leaves, roots, and wild agarwood of *A. sinensis*, with *Colletotrichum* as the generally dominant species and *Fusarium* as the specifically dominant species in agarwood. Xu [45] compared the fungi from the leaves of healthy *A. sinensis* trees and *Fusarium* sp.-inoculated trees, and found that *Collectrotrichum* was dominant in both types of leaves, but in drastically different compositions. Zhang et al. [48] isolated 42 fungi from the resin-forming section and the healthy xylem of *A. sinensis*, and evidenced that the genus of *Acremonium* was dominant in the healthy xylem, and the genus of *Penicillium* was dominant in the resin-forming section. In this study, a total of 110 fungi were isolated, and the species of *L. theobromae* was determined to be dominant in each of the four woody layers. The different fungal populations and dominance might be attributed to different habitats and growth conditions of *A. sinensis* trees. *A. malaccensis* is another agarwood-producing species. It was found that *Alternaria, Cladosporium, Curvularia, Fusarium, Phaeoacremonium*, and *Trichoderma* were found to be members of the agarwood community in *A. malaccensis* [32]. Additionally, it was to note that *Lasiodiplodia* species was once identified from the wounded samples of *A. malaccensis* from a natural forest in West Malaysia [27]. Fungi, for the first time, were identified simultaneously from all four layers during the agarwood-forming process in the present study. The fungi number isolated from the AL was much more than from the TL and NL, and even more than from the DL. Similar phenomena have been previously reported, where the fungi number in the resin-forming section was shown to be larger than that in the healthy xylem section [48]. The biochemical components in agarwood have anti-bacterial and anti-fungal activities, so that damage to trees can be lowered as much as possible. This explains why less fungi were found in the inner layers of TL and NL. Under the great demand for agarwood, the anti-fungal agarwood components, such as sesquiterpenes and phenylethyl chromone derivatives, give rise to a critical issue: Is the metabolism of certain fungi or the whole metabolic intensity of fungi in the AL associated with agarwood quantity and quality?

Lasiodiplodia theobromae is a common pathogen in the tropics and subtropics, and attacks more than 280 species of plants [2, 6, 7]. Its infection is frequently associated with stem, canker, dieback, and root rot disease [22, 37, 38]. The endophytic strains of *L. theobromae* have been found to produce jasmonic acid (JA), a kind of plant-growth inhibitor. JA and its derivates jasmoantes (JAs) are wound-related hormones and signal molecules commonly existing most plants. When applied exogenously, they can stimulate the defensive genes to increase the chemical levels of induced defenses [1, 17]. Agarwood forms only when the *A. sinensis* tree is wounded. Therefore, agarwood formation is also considered a result of *A. sinensis* defensive response. Sesquiterpenes and phenylethyl chromene derivatives are the main fragrant compounds of agarwood. Liao [23] confirmed that the JA and JAs signal molecules regulate the biosynthesis compounds of agarwood sesquiterpenes. Han [17] evidenced that *L. theobromae* produced JAs, thus leading to a significant increase of sesquiterpenes in *A. sinensis*. Krasnobajew and Helmlinger [20] reported that a strain of *L. theobromae* that transforms β-ionone into a large variety of metabolites mainly by degrading the side chain of the β-ionone molecule by a C2-unity. Matsuura et al. [25] obtained two metabolites (5*R*) and (5*S*) 5-hydroxylasiodiplodins from the culture filtrate of a strain of *L. theobromae*, which showed weak potato micro-tuber inducing activities. Yang et al. [47] isolated three hydroxylasiodiplodins from the mycelium extracts of *L. theobromae*, all of which showed the potato micro-tuber inducing activity. In the present study, the fermentation liquids of two isolates of *L. theobromae* were found to be capable of promoting agarwood formation. Up to now, a total of 88 isolates of *L. theobromae* have been obtained. We expect to identify more isolates with the agarwood formation-promoting activity or other valuable activities in future work.

Some fungal species have been investigated for their functions in promoting agarwood formation, such as *Aspergillus* spp., *Botryodyplodia* spp., *Diplodia* spp., *Fusarium* spp., *Penicillium* spp., *Trichoderma* spp., *Chaetomium globosum, Menanotus flavolives, Cytosphaera mangiferae*, and *Hypocrea jecorina* [4, 5, 12, 13, 26, 33, 35, 39, 41, 45]. In this study, two isolates of *L. theobromae* and one isolate of *F. solani* were evidenced to enable the promotion of agarwood formation. Although *Fusarium solani* (AF21) promoted agarwood formation at the initial stage, its effect was not stable in the later test; while *L. theobromae* (AF4 and AF12) still functioned 1 year later. In our previous report, the agarwood induced by the fermentation liquid of another isolate of *L. theobromae* showed similar composition and anti-fungal activity of essential oil to wild agarwood [50]. The performance comparison between AF12 and AF4 is under way in our lab. Although increasingly more fungi have been identified to be related with agarwood formation, the underlying mechanism of fungal participation in agarwood formation remains unclear. The present study may be helpful to the future development of agarwood-inducing techniques and the exploration of fungal functions in promoting agarwood formation.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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