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The fungal strain promotes rapid agarwood resin production with medicinally accepted agarotetrol level

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A R T I C L E I N F O

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A B S T R A C T

Agarwood, a highly valued, fragrant, dark, resinous heartwood, commands an estimated market value of over US $200 million. With the increasing market demand for agarwood, artificial induction methods have been a subject of study. Fungal induction is one of the methods that can effectively induce agarwood formation and is eco-friendly to the environment and humans. This study, however, brings a new perspective by focusing on identifying and screening fungal strains capable of rapidly inducing agarwood formation in *Aquilaria sinensis*. Pinhole-infusion technique (PIT) was used as a pre-experiment, and *Fusarium solani* (GDA-HC01) was the most effective strain among 12 tested fungal strains. A subsequent experiment using the Agar-Wit method was used to confirm *Fusarium solani* (GDA-HC01), and results showed *Fusarium solani* (GDA-HC01) can induce agarwood resin containing 1.4 times the agarotetrol content required by the Chinese Pharmacopeia within six months. Other strains, *Lasiodiplodia pseudotheobromae* (YNA-D3) and *Lasiodiplodia theobromae* (YNA-1C2), also showed some induction ability, while other strains had minimal effects. This is the first report demonstrating the rapid and consistent production of agarwood with medicinally accepted agarotetrol level in *Aquilaria sinensis* using *Fusarium solani* (GDA-HC01). With its potential for market application, this discovery is a significant step forward in the field and a valuable contribution to developing a sustainable green economy, offering a promising future for agarwood production.

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1. Introduction

Agarwood is a dark resin heartwood, mainly produced by the plant genera *Aquilaria* Lam. and *Gyrinops* Gaertn. (Thymelaeaceae Juss.) (Azren et al., 2018; Chen et al., 2018; Wang et al., 2018, 2019; Xu et al., 2016). Agarwood has a unique fragrance produced by its rich secondary metabolites, sesquiterpenes, and chromones (Cui et al., 2013; Rasool & Mohamed, 2016; Sen et al., 2017). It is often traded as incense, carvings, and jewelry, and the essential oil of agarwood is used in high-grade perfume (CITES, 2022; Liu et al., 2013; Monggoot et al., 2017; Ngadiran et al., 2023). Due to the small amount of agarwood collected in the wild and its unique fragrance, agarwood has a high economic value in the global market (Azren et al., 2018; Niego et al., 2023; Wang et al., 2018). At present, the global market of agarwood essential oil is estimated to be worth more than $200 million (Ngadiran et al., 2023; Niego et al., 2023).

Nevertheless, agarwood possesses significant medicinal value in addition to its other attributes. Agarwood plays important roles in traditional medicine in Arabia, China, and India, as well as in modern pharmacology (Du et al., 2022a; Liao et al., 2018; Liu et al., 2013; Ngadiran et al., 2023; Wang et al., 2018). Takamatsu and Ito (2020) proposed that agarotetrol is a characteristic substance of medicinal-grade agarwood. Chinese Pharmacopoeia (2015, 2020) has set agarotetrol as the main standard for testing whether agarwood has a medicinal value, i.e., if the agarotetrol content in the agarwood reaches 0.10%, it indicates the agarwood has a medicinally accepted agarotetrol level. In China, only two species of agarwood-producing trees, *Aquilaria sinensis* (Lour.) Spreng. and *A. yunnanensis* S. C. Huang are currently distributed, of which the main source of agarwood in China is *A. sinensis* (Chinese Pharmacopoeia, 2015, 2020; CITES, 2022; Tibpromma et al., 2021).

The natural formation of wild agarwood is slow, and yields are low because it unexpectedly occurs in over 20 years old trees as a defense mechanism against external damage (Du et al., 2022a; Liu et al., 2013; Ngadiran et al., 2023; Wang et al., 2018). Due to the extremely high economic value and strong market demand for agarwood, artificial induction methods have developed accordingly. Early physical methods involved using different tools to damage trees, which were simple and cost-effective, but required a significant amount of labor and time, resulting in low yields (Azren et al., 2018; Ngadiran et al., 2023; Tan et al., 2019; Wang et al., 2018). Later, chemical and biological methods were introduced, which involved creating wounds on trees and injecting inducers in different ways (Azren et al., 2018; Chen et al., 2017; Ngadiran et al., 2023). Chemical methods can quickly produce high-quality agarwood, there are some common compounds used to trigger the formation of agarwood, *viz*. jasmonic acid, sulfuric acid, acetic acid, and alcohol, but the chemicals injected into the trees may be released back into the environment, causing water and soil pollution, causing safety issues (Du et al., 2022a; Liu et al., 2013; Ngadiran et al., 2023; Wang et al., 2018; Zhang et al., 2012). The biological induction method most commonly reported by fungi as induction factors, and fungal induction is considered effective; in recent years, some research has also been conducted on bacteria as induction factors (Du et al., 2022a; Fitriasari et al., 2021; Ngadiran et al., 2023; Wang et al., 2018). The use of bacteria as inducers has not been extensively studied; the methods and technologies are still immature, and only a few bacteria have been reported to promote the production of some characteristic compounds in agarwood, e.g., *Bacillus* sp., *Bacillus pumillus*, and *Pseudomonas* sp. (Fitriasari et al., 2021). Fungi which is environmentally friendly and can produce high-quality agarwood resins similar to wild agarwood, although the results may vary depending on the fungal strain used (Du et al., 2022a; Mohamed et al., 2014; Mohammed et al., 2021; Ngadiran et al., 2023). The fungal induction method was first proposed by Tunstall in 1929 (Gibson, 1977) and scientists paid more attention to it with the development of science and technology. To date, 34 fungal genera have been used to induce agarwood production (Du et al., 2022a; Ngadiran et al., 2023), out of which, the most reported are *Fusarium*, followed by *Lasiodiplodia*, then, *Aspergillus* and *Botryosphaeria*. *Fusaruim solani*, *Cunninghamella bainieri*, and *Lasiodiplodia theobromae* are commonly reported to be used in the fungal inoculation process (Rasool & Mohamed, 2016). *Fusarium solani* was found to be the most effective fungus in inducing agarwood resin (Faizal et al., 2022).

There are two most common fungal induction methods, *viz*., the pinholes-infusion technique (PIT) (Tian et al., 2013) and the Agar-Wit technique (Liu et al., 2013). The pinhole-infusion technique involves injecting the inducer using a syringe, which is simple and easy to complete, and consumes a small amount of inducer (Tian et al., 2013). For example, Faizal et al. (2020) used a DeWalt® bore injector to drill holes and injected *Fusarium solani* inoculant into the *Gyrinops versteegii* trunk to induce resin formation. The Agar-Wit technique involves transporting the inducer using an infusion device, which is low cost, high yield, and easy to operate and is the most widely used artificial fragrance technology in agarwood-producing regions (Liu et al., 2013). For example, Zheng et al. (2019) induced the production of agarwood resin by injecting fungal inducers (*Fusarium solani* and *Lasiodiplodia theobromae*) into *Aquilaria sinensis* using the same method as Agar-Wit.

We, as a collective of researchers, are tackling the challenge of precisely screening effective fungal species. In the forest environment where agarwood-producing trees grow, hundreds or even thousands of microorganisms are on the surface and inside the trunk and bark. It is difficult to screen out effective agarwood-inducing fungi from such a large microbial library. This study is based on collective previous research and has screened some potential fungal strains for validation experiments. We also mentioned several previous publications that provide details about agarwood resin-inducing fungi (Faizal et al., 2020; Ngadiran et al., 2023; Wang et al., 2018; Zheng et al., 2019). Our research aims to support and identify the use of biological inducers to reduce and avoid adverse effects on the environment, livestock, and human health.

In this study, we selected the PIT method (Tian et al., 2013) to test the induction ability of agarwood resin from 12 selected strains. The fungi selected for this study were isolated from different parts of one of the agarwood-producing trees, *Aquilaria sinensis,* and were selected based on previous relevant reports. However, in previous studies, parallel experiments and analyses were not conducted on multiple strains. Therefore, this study analyzed the different induction effects and important agarwood characteristic compounds of the selected fungal strains. *Fusaruim* species have been used in the inoculation process (Akhsan et al., 2015; Faizal et al., 2017, 2020; Subasinghe et al., 2019; Zheng et al., 2019); *Fusarium solani* is considered the most effective fungus in promoting the production of agarwood resin (Herath & Jinendra, 2023; Turjaman et al., 2016, chap. 4), accompanied by the production of characteristic agarwood compounds (Faizal et al., 2020).

This study used fungal inoculation to induce agarwood production, addressing a key research gap: the lack of strains that can promote the production of agarwood that meets medicinal standards in a short time. This variability hampers the large-scale and reliable production of agarwood. The main objectives are to identify a fungal strain that consistently and efficiently promotes agarwood resin formation while ensuring the product meets medicinal standards, a task that is urgently needed in the field. The study highlights the biological significance of developing scalable, efficient methods for sustainable agarwood production by focusing on these objectives. Understanding the interactions between fungal strains and host trees is essential for improving induction techniques. The detailed processes, data, and analyses emphasize the potential impact on future agarwood cultivation.

2. Materials and methods

2.1. Pre-experiment - screening and identifying the most effective fungal strain for agarwood resin induction

2.1.1. Isolation and identification of the agarwood associated fungal strains

Twelve fungal strains were isolated from the agarwood resin parts, healthy leaves, and branches of agarwood-producing trees (*Aquilaria sinensis*) in Yunnan and Guangdong provinces (Table 1). The strains were isolated according to Du et al. (2022b), and pure cultures were used for genomic DNA extraction and deposited at Guizhou Medical University Culture Collection (GMBCC) in Guiyang, China.

These fungal strains were identified according to the description of Du et al. (2022b). Fungal mycelia on potato dextrose agar (PDA) aged one week were used to extract DNA and amplify polymerase chain reaction (PCR). The PCR products were purified and sequenced by Sangon Biotech Co., Kunming, China. The quality or chromatogram of the internal transcribed spacers (ITS) sequences obtained in the present study was checked in BioEdit v.7.2.6.1 (Hall, 1999), and the forward and reverse sequences were spliced with Geneious 9.1.8 (Kearse et al., 2012). The spliced sequences were blasted in GenBank (https://www.ncbi.nlm.nih.gov) for preliminary identification (Table 1). All newly generated sequences in this study were deposited to GenBank (https://www.ncbi.nlm.nih.gov) (Table 1).

2.1.2. Agarwood-producing tree

Thirteen healthy eight-year-old agarwood-producing *Aquilaria sinensis* trees were selected from agarwood plantations (Yunnan Yuanjiang Qinan Chenxiang Agricultural Technology Development Co., Ltd.) in Yunnan Province, China. Four healthy branches, each with a diameter ranging from 3 to 5 cm (from the first branch to the central branch), were selected from each tree for experimentation (modified from Faizal et al., 2020).

2.1.3. Determination of agarwood production

2.1.3.1. Preparation of fungal fermentation broth. Fungal fermentation broths were made according to the method described by Tibpromma et al. (2021). Fungal cultures were divided into small pieces (0.4 cm diam.) by sterilized straws; five pieces were placed in a conical flask containing 100 mL of sterile malt extract medium broth (MEB) (autoclaved at 121 °C, 20 min and kept at room temperature 25 ± 2 °C to cool it down). The conical flask mouth was sealed with cotton, and then the cotton was covered with tin foil to avoid contamination by other microorganisms. Finally, the conical flasks were incubated on a shaker (120 rpm, 28 °C) for five days in the darkness. Five days later, the mycelium blocks in the flasks were stirred by the sterilized glass rod and filtered by a filter to obtain fungal fermentation broth.

2.1.3.2. Inoculation method of fungal fermentation broth. This study used pinhole-infusion technology (PIT) to induce the production of agarwood (Tian et al., 2013). Thirteen healthy trees were selected for the experiment, with 12 trees as the experimental group labeled in order: A, B, C, D, E, F, G, H, I, J, K, and L. The 13th tree was the control group, marked as CK. Four branches are selected in each tree, and three holes (0.5 cm wide and 1–3 cm deep) are drilled on each selected branch using a disinfection drill. Three holes on each branch are evenly spaced (5 cm) and arranged in a straight line. In the experimental group, 12 different fungal fermentation broths were inoculated with 1 mL, thrice per hole, using sterile syringes for the experiment group (A-L). The control group was only drilled holes and was not inoculated with fermentation broth. Later, plastic films were used to seal the holes to avoid contamination by other microorganisms.

2.1.3.3. Agarwood analysis

2.1.3.3.1. The physical properties of agarwood. The total duration of this experiment was 12 months; one branch of each tree was cut off and observed every three months. The formation area and color of agarwood resin are crucial indicators of its quality. Typically, a larger formation area and a darker resin color suggest superior quality. The measurement and comparison of the formation area of agarwood resin forms were meticulously checked with a ruler tool, underscoring the precision and accuracy of the assessment. In contrast, agarwood resin's color observation and contrast were carried out through active visual engagement.

2.1.3.3.2. Detection of the content of three chromones in agarwood. Twelve-month-old agarwood samples were collected from each tree. All

Table 1
Collection site information and ITS Blast results of 12 *Aquilaria sinensis* associated fungi used in the present study.

Fungal isolate	Collection site	Collection date	Plant Tissue (isolation part)	Culture collection number	GenBank accession number	BLAST search results				
						Closest match	Isolate number	GenBank accession number	Identity	Query coverage
GDA-2A9	Guangdong	October 2020	Agarwood resin	GMBCC1190	PQ573370	*Lasiodiplodia pseudotheobromae*	FV-13 69 DSM 56A	OR398670	99.94%	90%
GDA-3A25	Guangdong	October 2020	Agarwood resin	GMBCC1191	PQ573371	*Fusarium proliferatum*	KT207283	KT207283	100%	96%
GDA-3A26	Guangdong	October 2020	Agarwood resin	GMBCC1192	PQ573372	*Trichoderma harzianum*	SF_752	MT530028	100%	95%
GDA-3B17	Guangdong	October 2020	Agarwood resin	GMBCC1193	PQ573373	*Daldinia eschscholtzii*	NQU283	MN368169	99.96%	97%
GDA-HC01	Guangdong	June 2022	Agarwood resin	GMBCC1194	PQ573374	*Fusarium solani*	YZM1	KY245947	99.91%	92%
YNA-1B2	Yunnan	September 2021	Agarwood resin	GMBCC1195	PQ573375	*Botryosphaeria fusispora*	GBLZ17BO-001	MN540670	100%	90%
YNA-1C1	Yunnan	September 2021	Agarwood resin	GMBCC1196	PQ573376	*Trichoderma harzianum*	NTOU 4300	MZ423061	99.94%	95%
YNA-1C2	Yunnan	September 2021	Agarwood resin	GMBCC1197	PQ573377	*Lasiodiplodia theobromae*	CDFA145	KY229166	99.92%	97%
YNA-2C5	Yunnan	September 2021	Agarwood resin	GMBCC1199	PQ573378	*Trichoderma koningiopsis*	DAOM 233971	EU280131	99.88%	96%
YNA-A18	Yunnan	November 2020	Healthy leaf	GMBCC1200	PQ573379	*Aspergillus niger*	HC2	OR534536	100%	90%
YNA-A73	Yunnan	November 2020	Healthy leaf	GMBCC1201	PQ573380	*Aspergillus niger*	S2596	MG590099	100%	90%
YNA-D3	Yunnan	November 2020	Healthy branch	GMBCC1202	PQ573381	*Lasiodiplodia pseudotheobromae*	B0271	KM006441	100%	94%

agarwood resins from each sample were cut into small pieces and mixed to make it homogenize, and then 0.2 g of each sample was selected for testing. The content of agarotetrol, 2-[2-(4-methoxyphenyl)ethyl]chromone (2-MC), and 2-(2-phenylethyl)chromone (2-PC) was detected by high-performance liquid chromatography (HPLC) following the established protocol (Chinese Pharmacopoeia, 2020; Quality Grade of Agarwood, 2017). According to the Chinese Pharmacopoeia (2020), a content of agarotetrol greater than 0.10% indicates that agarwood has medicinal value.

2.1.3.4. Identification of the fungal strains occurring during the agarwood production. To confirm whether the original inoculated strain induced the fungi from agarwood samples, each sample collected every three months was re-isolated. The isolation and identification methods were followed according to the methodology described in 2.1.1. ITS sequences were used to compare with the sequences of the original strains separately.

2.2. Extended experiment - agarwood production by Fusarium solani (GDA-HC01)

2.2.1. Source of fungal strains

The best strains identified and screened during the pre-experiment were used for further experiment expansion.

2.2.2. Agarwood-producing tree

The artificial plantation of agarwood-producing trees was the same as 2.1.2. The experiment was conducted on 8-year-old trees with a diameter of 15–20 cm (nine trees), which were healthy (no pests and diseases) and in good growth conditions in a tropical monsoon climate. According to the plantation, in 8-year-old trees, a diameter of 15–20 cm is a common size to conduct the experiment.

2.2.3. Determination of agarwood production

2.2.3.1. Preparation of fungal fermentation broth. The fungal fermentation broths were the same as mentioned in section 2.1.3.1, prepared according to the method described by Tibpromma et al. (2021). After five days of cultivation; they were obtained.

2.2.3.2. Inoculation method of fungal fermentation broth. Two standard fungal inoculation methods were selected as a comparison, aiming to find a fast and efficient way to induce agarwood for six months. The two methods used in this experiment were the pinholes-infusion technique (PIT) (Tian et al., 2013) and the Agar-Wit technique (Liu et al., 2013). Nine trees were divided into three groups (three trees in each group) and tested in the PIT, Agar-Wit, and control groups. Each tree was drilled with three holes (0.5 cm diam., 6–8 cm deep) using a sterilized electric drill.

In the PIT group, each tree was arranged in a vertical straight line, with the first hole at a height of 50 cm above the ground, and the distance between each hole is 8 cm. Fungal fermentation broth was injected into the holes by using sterile syringes, injecting 1 mL into each hole three times (total of 3 mL/hole) and plastic films were used to seal the holes to avoid contamination by other microorganisms.

In the Agar-Wit group, the three holes on each tree were arranged in a vertical spiral pattern, with the first hole located 30 cm above the ground and a distance of 10 cm between each hole. The Agar-Wit groups were injected with fungal fermentation broth into the holes using infusion bags and tubes. First, 500 mL of fermentation broth was put in each infusion bag, and each infusion bag was equipped with three infusion tubes. Then the infusion tubes were inserted into the holes respectively. Finally, the flow rate was adjusted to be input into the tree trunk within 3–5 days. Finally, plastic films were used to seal the holes to avoid contamination by other microorganisms.

The control groups were only drilled holes and were not inoculated with fermentation broth. Plastic films were used to seal the holes to avoid contamination by other microorganisms.

2.2.3.3. Agarwood analysis. The inspection method for inoculation results was the same as in section 2.1.3.3. The results were checked after six months.

2.2.3.4. Statistical analysis. To evaluate the effect of different treatments on agarotetrol, one-way ANOVA was used. This experiment set up three treatment groups: The Agar-Wit, PIT, and the control. The analysis of variance was conducted using IBM SPSS v. 27 statistical software, with a significance level set at 0.05. Use one-way analysis of variance to test the differences in agarotetrol content between groups. At the same time, to visually demonstrate the impact of different processing methods on agarotetrol content, this study used IBM SPSS v. 27 statistical software to draw box plots.

2.2.3.5. Identification of the fungal strains occurring during the agarwood production. To confirm whether the original inoculated strain induced the fungi from agarwood samples, each sample collected every six months was re-isolated. The isolation and identification methods followed the methodology described in section 2.1.1. ITS sequences were used to compare with the sequences of the original strains, separately.

3. Results

3.1. Isolation and identification results of the agarwood associated fungal strains

Twelve fungi strains isolated from *Aquilaria sinensis* are listed in Table 1 with relevant information (collection site, date, and isolation part). The preliminary identification based on ITS BLAST search in NCBI resulted in *Aspergillus niger* Tiegh. (Identity: 100%, YNA-A73 and 100%, YNA-A18), *Botryosphaeria fusispora* Boonmee, Jian K. Liu & K.D. Hyde (identity: 100%, YNA-1B2), *Daldinia eschscholtzii* (Ehrenb.) Rehm (Identity: 99.66%, GDA-3B17), *Fusarium proliferatum* (Matsush.) Nirenberg (Identity: 100%, GDA-3A25), *F. solani* (Mart.) Sacc. (Identity: 99.81%, GDA-HC01), *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous (Identity: 99.64%, GDA-2A9 and identity: 100%, YNA-D3), *L. theobromae* (Pat.) Griffon & Maubl. (Identity: 99.82%, YNA-1C2), *Trichoderma harzianum* Rifai (identity: 100%, GDA-3A26 and 99.84%, YNA-1C1) and *T. koningiopsis* Samuels, Carm. Suárez & H.C. Evans (identity: 99.68%, YNA-2C5). In addition, YNA-D3 and GDA-2A9 were identified as *Lasiodiplodia pseudotheobromae*; even though they were isolated from different host parts (YNA-D3 from a healthy branch while GDA-2A9 from agarwood resin part).

3.2. Analysis of induction results of 12 fungal strains on agarwood resin (pre-experiment)

Notably, previous studies revealed that fungal infections accelerate the process of agarwood formation when compared to the traditional method of inducing agarwood, and these fungi were isolated from agarwood resin or agarwood-producing trees (Ngadiran et al., 2023). Therefore, this study selected 12 fungal strains isolated from *Aquilaria sinensis* to investigate their agarwood resin formation potential.

After 12 months, 12 *Aquilaria sinensis* trees inoculated by fermentation broths of 12 fungal strains formed black-brown layers of agarwood resin around the hole. All groups in the pre-experiment showed their ability to induce agarwood resins, including the control group; the results are meticulously recorded in Table 2 and visually represented in Fig. 1. According to the comparison of the colors of the samples collected in the 12^{th} month, the 12 groups are arrayed in descending order from darken to clarity pigment: G > A > B > C > F > I > J > D > K > L > H >

Table 2
Agarwood induction results (Pre-experiment).

Agarwood tree inoculated by fungal isolate	The 3rd month			The 6th month			The 9th month			The 12th month						
	Original strain re-isolated	Wound healing	Average width (cm)	Original strain re-isolated	Wound healing	Average width (cm)	Original strain re-isolated	Wound healing	Average width (cm)	Original strain re-isolated	Wound healing	Average width (cm)	Agarotetrol content (%)	Improvement rate	Content of 2-MC and 2-PC (%)	Improvement rate
YNA-D3 (group A)	Y	N	4.12	Y	N	4.8	Y	N	6	Y	N	6.5	0.0003%	0.4	0.0164%	0.9
YNA-1C1 (group B)	Y	Y	0.7	Y	Y	1.1	Y	Y	0.68	N	Y	0.5	0.0004%	0.5	0.0037%	0.2
GDA-2A9 (group C)	N	Y	0.36	Y	Y	1.5	Y	Y	1.1	Y	Y	0.8	0.0025%	3.6	0.0326%	1.7
YNA-A18 (group D)	Y	N	1.34	Y	Y	1.15	N	Y	0.7	N	Y	0.6	0.0009%	1.2	0.0098%	0.5
YNA-1B2 (group E)	Y	N	0.24	Dead	N	0.4	Dead	N	0.35	Dead	N	0.4	/	/	0.0043%	0.2
GDA-3A25 (group F)	N	Y	0.35	N	Y	0.3	N	Y	0.5	N	Y	0.48	0.0014%	2.0	0.0060%	0.3
GDA-HC01 (group G)	Y	N	8.5	Y	N	9.8	Y	N	12.3	Y	N	13.5	0.0267%	37.7	0.0710%	3.8
YNA-1C2 (group H)	Y	N	3.5	Y	N	4.5	Y	N	4.8	Y	N	2.7	0.0040%	5.6	0.0203%	1.1
YNA-2C5 (group I)	N	N	0.22	N	N	0.55	N	N	1.2	Y	Y	0.8	0.0012%	1.7	0.0078%	0.4
GDA-3A26 (group J)	N	Y	0.23	Y	Y	0.4	N	N	1.1	N	N	0.78	0.0027%	3.8	0.0169%	0.9
GDA-3B17 (group K)	N	N	0.18	N	N	0.2	N	N	1.5	N	N	0.23	0.0013%	1.8	0.0040%	0.2
YNA-A73 (group L)	Dead	N	0.1	Dead	N	0.18	Dead	N	0.16	N	N	1.6	0.0067%	9.4	0.0083%	0.4
CK	/	N	0.34	/	N	0.36	/	N	0.41	/	N	0.46	0.0007%	1.0	0.0189%	1.0

Remarks: "Y" = Yes, "N" = No, "/" = Inapplicable, "CK" = Control group, "Dead" refers to this sample that had already died when it was collected, Average width = The average width that extends longitudinally along the xylem (sum of the upper and lower sides of the hole).

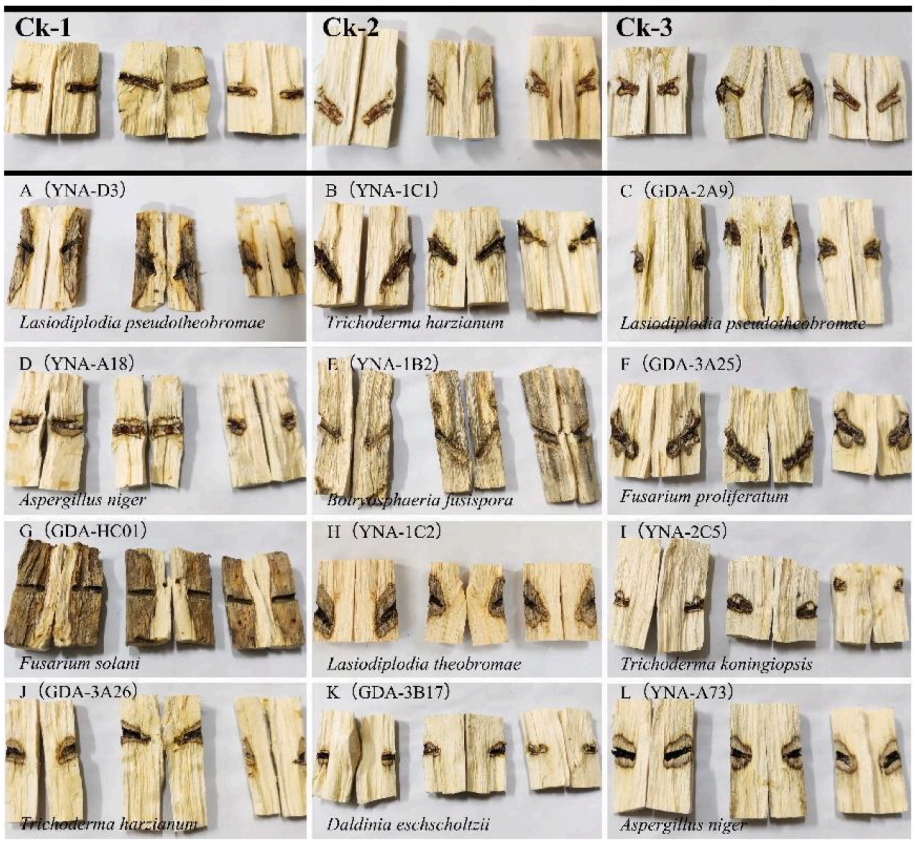


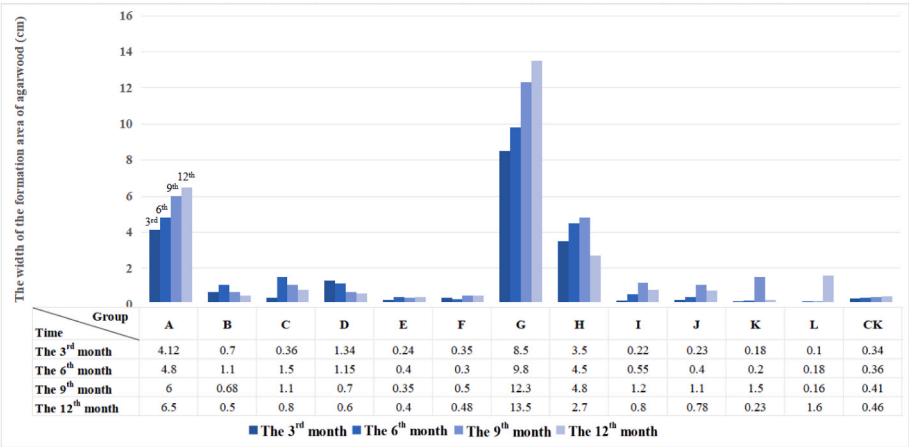
Fig. 1. Induction results of agarwood resin, after one year of inoculation with 12 different fungal fermentation broths. White is the normal color of the xylem, while brown to dark brown is the agarwood resin part. Ck–1,–2 and –3: Control groups. A–L: Group A–L. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

E. There are significant differences among them (Fig. 1).

Group G exhibited the most effective induction effect (Table 2 and Fig. 1). Its average growth width expanded to 8.5 cm by the third month, demonstrating a continuous increase over time, ultimately reaching 13.5 cm by the 12th month. Moreover, by the 12th month of compound testing, group G exhibited significantly medicinally accepted agarotetrol level and other two chromones (2-MC and 2-PC) content compared to the other groups. Specifically, its agarotetrol content was 37.7 times higher than that of the control group, and its 2-MC and 2-PC content was 3.8 times higher than that of the control group. Notably, the experimental observations for group G indicated the absence of wound healing or death of central tissue (around the hole). The original strain was successfully obtained during the re-isolation.

Regarding formation area and compound content (Table 2, Figs. 1 and 2), groups A and H showed more significant results than the other ten groups (including the control group). Neither group A nor group H manifested any discernible signs of wound healing; instead, the central regions exhibited pronounced desiccation and necrosis. Particularly, the observation of group H was noteworthy, wherein the area formed by 12 months witnessed a substantial reduction compared to the measurements taken in the ninth month.

The induction effects of the other six groups (B, C, D, E, J, and L) were not significantly different from those of the control group. All six experimental groups had wounds that healed over time, and three



Time \ Group	A	B	C	D	E	F	G	H	I	J	K	L	CK
The 3rd month	4.12	0.7	0.36	1.34	0.24	0.35	8.5	3.5	0.22	0.23	0.18	0.1	0.34
The 6th month	4.8	1.1	1.5	1.15	0.4	0.3	9.8	4.5	0.55	0.4	0.2	0.18	0.36
The 9th month	6	0.68	1.1	0.7	0.35	0.5	12.3	4.8	1.2	1.1	1.5	0.16	0.41
The 12th month	6.5	0.5	0.8	0.6	0.4	0.48	13.5	2.7	0.8	0.78	0.23	1.6	0.46

Fig. 2. Comparison of the agarwood resin formation area width collected in 12 groups and control groups at different periods.

groups showed wound healing and central tissue death (groups B, C, and E). The size of the area formed by agarwood in these groups remained unchanged or decreased over time.

In addition, groups D and L showed a mortality rate of 75% in all collections. A total of four branches were collected, of which three died. Fungal species of these two groups all belong to *Aspergillus niger*. While the other three groups (F, I, and K) did not obtain the original strains during the re-isolation.

Therefore, based on the pre-experiment results mentioned above, Group G showed significant positive results compared to other experimental groups (Table 2 and Fig. 1). Therefore, Group G strain *Fusarium solani* (GDA-HC01) was selected for further agarwood induction experiments.

3.3. Analysis of agarwood resin induction results of Fusarium solani (GDA-HC01) (extended experiment)

The pre-experiment results showed that group G (*F. solani* GDA-HC01) had the best effect, as it had a darker, wider resin area and higher chromone content compared to other experimental groups (Table 2 and Fig. 1); therefore, this strain was selected as the best strain for the extended experiment.

According to the six-month results, the growth width of agarwood infused with infusion bags in Agar-Wit is 2.2 times larger than that in PIT and 6.6 times larger than that in the control group (Table 3, Fig. 3). Regarding the colors, Fig. 3-B (control group) appears black, a color that only forms after the tissue necrosis. In contrast, Fig. 3-C (PIT) displayed brown resin, while Fig. 3-D (Agar-Wit) showed brown to black, brown resin. The darker color of Agar-Wit's resin compared to PIT's indicated higher quality. At the same time, in terms of texture, the resin content of PIT was relatively low, the oil lines were sparse, the oil distribution was uneven, the hand feel was light, it felt slightly rough, the woody feeling was more obvious, and the fragrance was weak and not long-lasting when burned; while, the Agar-Wit had a higher resin content, especially in the central part, with abundant internal oil and clear and full oil lines, a harder texture, a heavier hand feel, and a smoother texture; when burned, the smoke was light and soft and lasted longer.

The agarotetrol content in Agar-Wit is 3.4 times that of PIT and 216.6 times that of the control group, while the 2-MC and 2-PC content of Agar-Wit is 1.5 times that of PIT and 40.4 times that of the control group (Table 3). The structures of agarotetrol, 2-MC, and 2-PC are shown in Fig. 4, and the peak plot of High-performance liquid chromatography (HPLC) is shown in Fig. 5.

The statistical analysis results showed that there is a significant difference in the effect of different treatment methods on the content of agarotetrol ($F = 1220.2$, $p = 1.37 \times 10^{-14}$, far less than 0.05). The specific analysis is as follows:

Agar-Wit has the highest content of agarotetrol, with an average of 0.14%. The agarotetrol in this treatment group was significantly higher than that in other groups ($p < 0.001$), indicating that the Agar-Wit method has the strongest promoting effect on the synthesis of agarotetrol. PIT: The agarotetrol content was 0.04%, second only to the Agar-Wit method and significantly higher than the control group ($p < 0.001$). This indicates that the PIT method can also promote the synthesis of agarotetrol to a certain extent, but its effect is not as good as the Agar-Wit method. Control: Agarotetrol has the lowest content, averaging only 0.001%, which is almost negligible. The agarotetrol content in the control group was significantly lower than that in the treatment group ($p < 0.001$), indicating that the natural generation of agarwood resin is extremely limited under no treatment conditions. In summary, the Agar-Wit method has the strongest promoting effect on the synthesis of agarotetrol; in contrast, the PIT method, although less effective, is still significantly higher than the control group, indicating that it also has a certain effect on increasing the content of agarwood resin. The box plot (Fig. 6) further illustrates the significant differences among the three groups: the data distribution of the Agar-Wit group is completely higher than the other two groups, followed by the PIT group, and the Control group has the lowest and tighter distribution. These results indicate that there are significant differences in the induction effect of different fungal treatment methods on agarotetrol, with the Agar-Wit method being the most effective.

In addition, the agarotetrol content in Agar-Wit exceeds the Chinese Pharmacopoeia (2020) requirement (0.1%) by 1.4 times. From this, it can be seen that strain *F. solani* (GDA-HC01) can quickly induce agarwood through Agar-Wit within six months and meet the medicinally accepted agarotetrol level.

3.4. Analysis of fungal strains in the induction process of agarwood

In the results of strain re-isolation, all re-isolated strains were compared with the original strain in terms of bases, and the results showed that, except for the inevitable impact of peaks at both ends of the ITS gene, most were not different from the original strain. Some strains had a 1 or 2 bp base difference, which is less than 0.5%. Except for the original strains, other strains belonging to 20 genera were also isolated *viz.* *Acrocalymma* Alcorn & J.A.G. Irwin, *Alternaria* Nees, *Aspergillus*, *Camarosporium* Schulzer, *Colletotrichum*, *Crassiparies* M. Matsum., K. Hiray. & Kaz. Tanaka, *Daldinia* Ces. & De Not., *Deniquelata* Ariyaw. & K. D. Hyde, *Diaporthe* Nitschke, *Fusarium*, *Hypoxylon* Bull., *Lasiodiplodia*, *Medicopsis* Gruyter, Verkley & Crous, *Montagnula* Berl., *Mucor* P. Micheli, *Neoscytalidium* Crous & Slippers, *Penicillium* Link, *Phaeoacremonium* W. Gams, Crous & M.J. Wingf., *Pseudofusicoccum* Mohali, Slippers & M.J. Wingf. and *Xylaria*. *Lasiodiplodia* was the most prominent genus, accounting for 50% of all the above.

4. Discussion

This study conducted pre- and extended experiments to screen the best fungal strain and explore the most suitable inoculation method for inducing agarwood resin formation.

In the pre-experiment, 12 strains were selected to undergo PIT induction on the small branches of 12 trees. The pre-experiment results indicated that all available strains can induce agarwood formation, but their effects vary greatly. This PIT causes minimal damage to the host and uses less inoculum, so it is the most appropriate method for inoculation experiments on small branches. In this experiment, an agarwood-associated fungal strain (*Fusarium solani* GDA-HC01) showed the best result: an increase in the formation area of agarwood resin over time, and no signs of wound healing or tissue death around the wound were observed. Therefore, this strain was selected for the extended

Table 3
Comparison of agarwood induction results of different methods after six months.

Methods	Original strain re-isolated	Wound healing	Average width (cm)	Agarotetrol content (%)	Improvement rate	Content of 2-MC and 2-PC (%)	Improvement rate
Agar-Wit	Y	N	3.3	0.14%	216.6	0.26%	40.4
PIT	Y	N	1.5	0.04%	63.4	0.17%	26.2
Control	/	N	0.5	0.001%	1.0	0.01%	1.0

Remarks: "Y" = Yes, "N" = No, "/" = Inapplicable, Average width = Horizontal expansion average width.

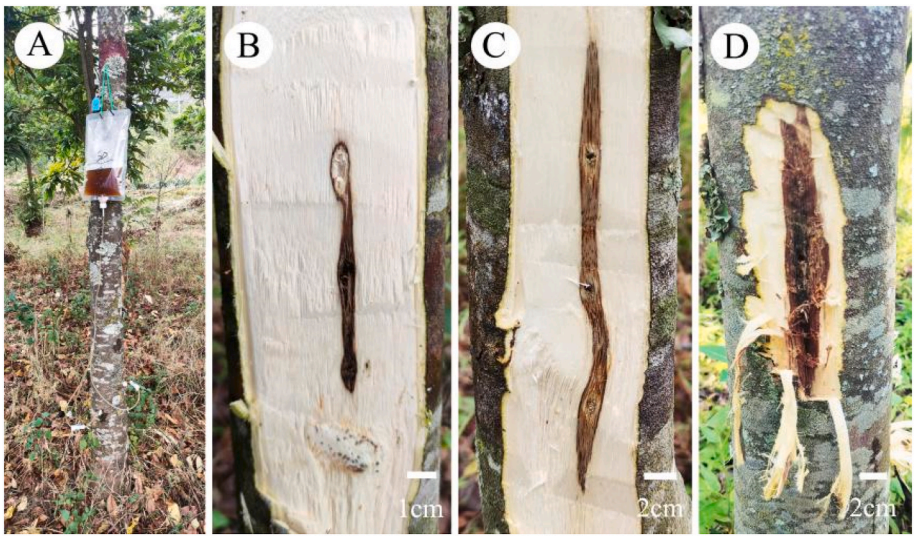


Fig. 3. The results of the extended experiment in the sixth month. (A) Field experiment site in the plantation. (B) Control group (the black part represents decaying tissue). (C) PIT. (D) Agar-Wit.

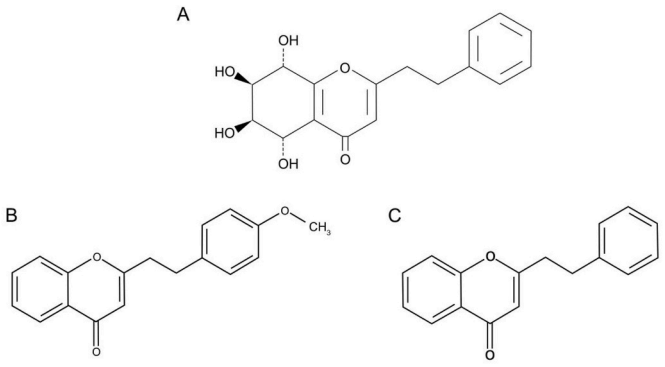


Fig. 4. Structures of agarwood chromones that were obtained in this study. (A) Agarotetrol. (B) 2-[2-(4-methoxyphenyl)ethyl]chromone (2-MC). (C) 2-(2-phenylethyl)chromone (2-PC).

experiments on tree trunks, and the results showed that after only six months, the content of agarotetrol in agarwood resin induced by the *F. solani* (GDA-HC01) was 1.4 times higher than the required standard.

Species of *Fusarium* are commonly reported as endophytic fungi or agarwood resin-associated fungi in agarwood-producing trees (Azren et al., 2018; Chhipa et al., 2017; Du et al., 2022a; Ngadiran et al., 2023). *Fusarium solani* has been proven to be an effective fungus in inducing the formation of agarwood resin in previous studies. For example, Faizal et al. (2020) reported that *Gyrinops versteegii* (Gilg.) Domke produced a large area of agarwood after being inoculated with a fungal solution of *F. solani* (strains GSL1 and GSL2) for three months, while the study did not detect the content of agarotetrol. In previous studies, Chen et al. (2018) reported that the content of agarotetrol induced by *Rigidoporus vinctus* (Berk.) Ryvarden could meet the medicinal standards in the sixth month. This study reports for the first time that qualified agarotetrol can be detected on induced wood after six months of inoculation with *F. solani* (GDA-HC01) on *A. sinensis*. These pieces of evidence indicate that this strain has a high potential in inducing agarwood resin formation and is expected to be developed into a highly efficient, low-cost, pollution-free, and environmentally friendly fungal inducer.

In addition, both group A (*Lasiodiplodia pseudotheobromae* strain YNA-D3) and group H (*L. theobromae* strain YNA-1C2) were able to significantly induce the production of agarwood resin; but both showed signs of central tissue necrosis, with group H being more severe. *Lasiodiplodia pseudotheobromae* was first reported to have the ability to induce agarwood production in this study. This may be worth further research.

Additionally, in groups F (*Fusarium proliferatum* strain GDA-3A11), I (*Trichoderma koningiopsis* strain YNA-2C5), and K (*Daldinia eschscholtzii* strain GDA-3B13), original strains were not obtained during re-isolations. The possible reason could be that these three strains could not settle in *A. sinensis* trees. This might be because those fungi are related to the inherent characteristics of the strain, host, temperature, humidity, and other factors. Therefore, further research is needed to explore why these three strains cannot settle on *A. sinensis* trees to induce agarwood resin.

Moreover, in this experiment, four experimental groups (groups B, C, D, and J) showed healing of tree wounds, and two groups (groups B and C) showed healing of tree wounds and central tissue death. These two situations produced small resin regions and did not accumulate more resins over time, resulting in gradual wound healing and less damage to the tree. Groups A and H experienced severe central tissue death without healing, causing certain damage to the tree, while E and L groups had a mortality rate of up to 75% for tree branches. However, group G did not show any sound healing or central tissue death; over time, it promoted the formation of more agarwood resin. Therefore, this study indicates that some strains (from groups B, C, D, and J) that have no effect on the tree or cause too weak damage have minimal damage to the tree, making it easy for the tree to produce callus tissue for wound healing. The strains (from groups A, E, H, and L) that cause too serious damage to the tree will cause the tree to die or wither around the inoculation hole, so neither of these strains is suitable for inducing agarwood production.

In the extended experiment, two methods *viz.* PIT and Agar-Wit were used to explore the suitable methods for fungal induction of agarwood. After six months, PIT significantly promoted the production of agarwood resin compared to the control group, although the agarotetrol content did not meet the required standard. In contrast, the Agar-Wit method not only significantly enhanced agarwood resin formation but also resulted in agarotetrol amount that meets the medicinally accepted agarotetrol level. This may be because Agar-Wit can inject a large amount of fungal fermentation broth (500 mL/tree) simultaneously, and a large amount of fungal fermentation broth is input into the lower base of the tree trunk, facilitating the colonization of the fermentation broth. Then, through transpiration, it continuously infects and induces the trees to accelerate the formation of a large amount of agarwood resin. However, the volume content of the fermentation broth used by Agar-Wit is relatively high, and further experiments are needed to determine the most suitable

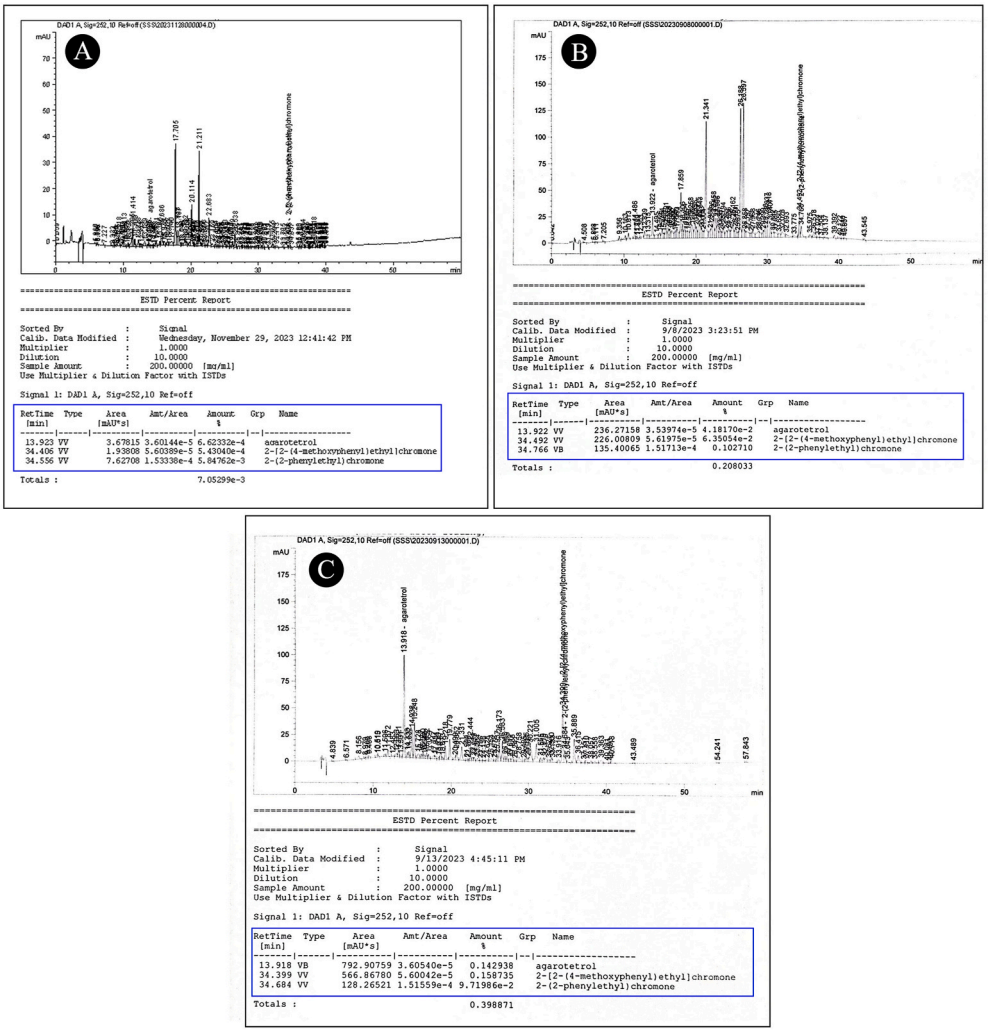


Fig. 5. High-performance liquid chromatography (HPLC) results of agarwood resin induced by three treatments. (A) Control group. (B) PIT. (C) Agar-Wit. The blue box indicates the content of three main compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

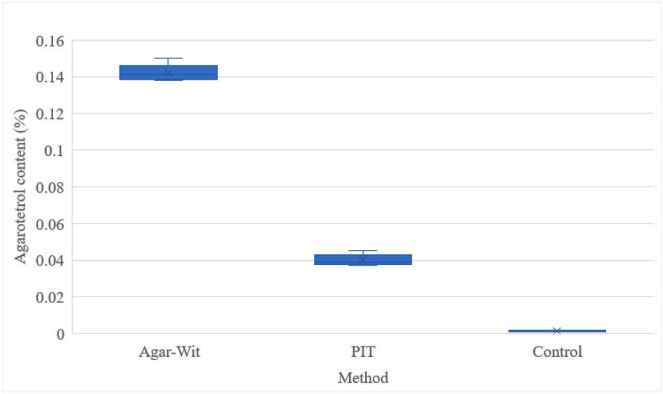


Fig. 6. The agarotetrol content under different treatments.

volume of fermentation broth for inoculation. In addition, using fermentation broth with different concentration gradients to determine the optimal concentration for agarwood induction is also a worthy topic for future research. Subsequent research should further focus on exploring other methods of fungal agent production to reduce costs, making it easier for pollution-free fungal inducers to be promoted and used in the market, and promoting sustainable development of the green economy.

The results of strain re-isolation showed that *Lasiodiplodia* is the most prominent genus except for the original strains. These fungi may contribute to the formation of agarwood resin, which is one of the many factors.

Our research has revealed significant differences in the induction results of the 12 strains through pre-experiments. These findings are closely related to the interactions between fungi and plants. The effects vary depending on the strain, and the difference in induction effect may be due to the different secondary metabolic pathways of different strains. These pathways produce different types and contents of secondary metabolites during growth, which play a crucial role in interacting with fungi and host plants. For strains with good induction effects, the secondary metabolites produced may be more conducive to activating genes related to agarwood formation in the host plant. The formation mechanism of agarwood is currently unclear and presents an exciting opportunity for further research. The data and strains provided in this study can assist subsequent research and fuel curiosity in this field.

The advantage of this study is the identification of an effective strain capable of inducing agarwood resin formation, which achieved significant results within six months. *Lasiodiplodia pseudotheobromae* was first reported to have the ability to induce agarwood production in this study.

However, some strains didn't have the ability to colonize on trees (*Fusarium proliferatum* strain GDA-3A11, *Daldinia eschscholtzii* strain GDA-3B13, and *Trichoderma koningiopsis* strain YNA-2C5), while, some strains caused severe tissue damage (*Aspergillus niger* strain YNA-A73 and *Botryosphaeria fusispora* strain YNA-1B2), restricting their applicability. Although the Agar-Wit method is applicable in this study, further research is needed to optimize inoculation volume and concentration to maximize efficiency and reduce costs.

The significance of this study lies in the potential for utilizing *Fusarium solani* and other strains for fungal induction. This approach can significantly shorten the formation cycle of agarwood and boost its yield while reducing dependence on chemical agents in production, thereby reducing environmental pollution and protecting soil microbial communities. To mitigate damage to the ecological environment, we can develop low-damage induction techniques and precise inoculation techniques when promoting the use of these strains in agarwood production. These technical techniques can improve the effective interaction between strains and agarwood trees by controlling the depth, dosage, and location of inoculation while reducing negative impacts on trees and the surrounding environment. Moreover, the application of modern biotechnology, such as gene editing, can enable strains to more accurately target specific tissues or cells within the agarwood-producing tree, thereby enhancing the induction efficiency of agarwood while reducing the risk of strain spread in the environment. To ensure the sustainability of these methods, it is crucial to stress the importance of regularly monitoring soil, air, and water quality during fungal inoculation. This will prevent strain spread or negative impacts on soil microbial communities. If necessary, soil disinfection or fungal control treatment can be carried out to maintain the stability of the surrounding ecosystem. At the same time, a corresponding ecological management system should be established, and strict operational norms and standards should be formulated. This will ensure that agarwood production does not cause excessive damage to the surrounding ecological environment, and will help us achieve sustainable production.

In this study, we successfully identified *Fusarium solani* (GDA-HC01) as an efficient fungal strain that induces agarwood resin formation, demonstrating its potential to meet and exceed medicinal standards in just six months. This discovery provides valuable insights for optimizing fungal inoculation to achieve sustainable and efficient agarwood production; and it has the potential to develop a low-cost, environmentally friendly inducer that can be widely applied in the industry. Future research will focus on optimizing inoculation volume and fermentation broth concentration to increase agarwood yield further while reducing costs and promoting green production in the agarwood industry.

5. Conclusions

The innovation part of this study is the discovery of *Fusarium solani* (GDA-HC01) with good agarwood resin induction potential, which was tested on *A. sinensis* in field experiments. The fungal strain *F. solani* (GDA-HC01) is patented under patent number 7067418 (https://pss-system.cponline.cnipa.gov.cn/conventionalSearch) (Du et al., 2024).

The conclusions are as follows:

1. *Fusarium solani* (GDA-HC01) can quickly and stably induce the formation of agarwood resin in *Aquilaria sinensis.* Within six months, the content of agarotetrol in agarwood resin can meet the medicinally accepted agarotetrol level, and it does not cause the tree's wound to heal or wither around the wound.
2. *Lasiodiplodia pseudotheobromae* (YNA-D3) and *L. theobromae* (YNA-1C2) significantly induced agarwood resin. This is the first report of *L. pseudotheobromae* that can induce agarwood production, but they cause the central tissue to wither.
3. *Aspergillus niger* (YNA-A73) and *Botryosphaeria fusispora* (YNA-1B2) showed direct withering of the tested branches, with a mortality rate of up to 75%. Therefore, we suggest that if *A. niger* and *B. fusispora* will be used to conduct relevant induction experiments on *A. sinensis* trees, it is recommended to conduct a pre-experiment on small tree branches first to avoid unnecessary and unpredictable damage to the trees.

CRediT authorship contribution statement

Tian-Ye Du: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Saowaluck Tibpromma:** Writing – review & editing, Visualization, Validation, Resources, Project administration, Funding acquisition, Data curation. **Kevin D. Hyde:** Writing – review & editing, Validation. **Yue-Hu Wang:** Writing – review & editing, Validation. **Putarak Chomnunti:** Writing – review & editing, Validation. **Ekachai Chukeatirote:** Writing – review & editing, Validation. **Wen-Hua Lu:** Writing – review & editing, Validation. **Ausana Mapook:** Writing – review & editing, Validation. **Dong-Qin Dai:** Writing – review & editing, Project administration, Funding acquisition. **Douglas S.A. Wijesundara:** Writing – review & editing, Validation. **Abdallah M. Elgorban:** Writing – review & editing, Validation. **Nakarin Suwannarach:** Writing – review & editing, Validation. **Jaturong Kumla:** Writing – review & editing, Validation. **Ihab M. Moussa:** Writing – review & editing, Validation. **Hao-Han Wang:** Writing – review & editing, Validation. **Samantha C. Karunarathna:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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