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Optimization and induction effect evaluation of complex inducer of *Aquilaria sinensis* based on factorial design

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Agarwood is a kind of valuable traditional Chinese medicine. However, natural agarwood has a long formation cycle and is in short supply. Herein, the complex inducer of artificial agarwood was screened and optimised based on design of experiments (DoE). Single factor design experiments were conducted to evaluate the effects of different concentrations of methyl jasmonate (A-MeJA), formic acid (B-FA) and *Botryosphaeria rhodina* A13(C-A13) on the in vitro branches of *A. sinensis* at different induction times (D-Time). The 2^k factorial design was used to further optimise the screening of the complex inducer. Single factor experiments determined the concentration of the inducers acting on the branches of *A. sinensis*. The order of the four factors obtained by 2^k factorial experiment was D-Time > A-MeJA > B-FA > C-A13. The optimal complex inducer was 1%MeJA + 1%FA + A13. Verification through an in vivo field experiment of 18 batches of *A. sinensis*, artificial agarwood met the requirements of the Pharmacopoeia of People's Republic of China 2020 (ChP 2020), an indication that the complex inducer could efficiently promote the formation of agarwood (in 9 months). These findings provide essential data and new ideas for the efficient induction of agarwood and the production of high-quality substitutes for natural agarwood.

Keywords Agarwood, Complex inducer, Factorial design, Methyl jasmonate, Sesquiterpenes

Herbal and medicinal plants play a vital role on the life of human beings and have unique compartment in their lifestyles. humanity faces numerous persistent diseases and illnesses, for which medicinal and herbal plants offer promising therapeutic options. Evidence for the effectiveness of herbal drugs has been obtained using extracts from aromatic and medicinal plants including essential oils, alcoholic, hydrolyzed and other solvent-based extracts along with fruit juices and extracts obtained from resins[1]. The plants *Aquilaria* spp. belong to the Thymelaeaceous family and are the typical wounding-induced medicinal plants primarily distributed throughout Southeast Asia. Depending on the region agarwood (also called Chen-Xiang, agar, gaharu, eaglewood, or aloeswood) is a resinous part of the non-timber *Aquilaria* tree. The chemical composition of agarwood mainly consists of sesquiterpenoids, 2-(2-phenylethyl) chromones and aromatics. Agarwood is a precious product for perfumes, incenses, and traditional Chinese medicine. In China, agarwood originates from aromatic resinous portion of *A. sinensis* (Lour.) Gilg and is generally used as a spiritual healing drug, digestive, sedative, and antiemetic drug[2–4]. Through its medicinal value, aroma healing and cultural and economic significance, agarwood contributes to human health and quality of life in many ways. With the deepening of modern scientific research, the potential of agarwood is expected to be further explored, bringing more benefits to human health.

In general, the fragrant resin is not produced in healthy *A. sinensis* and requires a long period of time to be produced under natural conditions (e.g., moth-eaten, thunderstruck, typhoon, and so on)[5–7]. Over decades, people pondered on how natural agarwood is formed. Indeed, agarwood is a typical injury-induced plant. Common physical and traditional agarwood-inducing methods are trunk wounding through a knife cut, hammering nails into *A. sinensis* tree trunks, drilling, and heat shock, all of which are mechanical injuries;

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and the formation is usually in the proximity of the wounded or decaying parts of the trunk[8,9]. Chemicals are potent agarwood inducers. Examples of these chemicals can be phytohormone such as hydrogen peroxide (H_2O_2), MeJA, and salicylic acid (SA), or chemicals such as sodium chloride (NaCl), FA, and so on[10–13]. Research has shown that exogenous MeJA can induce the formation and accumulation of sesquiterpenoids and when combined with SA in *A. sinensis*, can improve the stress tolerance[14–16]. The chemical inducer acts by causing severe damage to the cellular structure of the trunk of the *Aquilaria* spp., and as a result, callusing to cover up the wound cannot take place. Physical and chemical damage can weaken the *A. sinensis* and in turn become vulnerable to fungal infection[13,14,17]. Over the years, many researchers believe that microbes play an important role in agarwood formation and thus have isolated endophytic fungi from agarwood zones[18–20]. Furthermore, biological inoculation using specific fungi as inoculants has been conducted, and some of these fungi are members of the genera *Fusarium*, *Colletotrichum*, *Botryosphaeria* and many others[13,21–25]. However, natural agarwood is formed by four induction modes: physical injury, chemical damage, fungal infection, and comprehensive stimulation[26].

Main compounds in agarwood are sesquiterpenes and 2-(2-phenylethyl) chromones, and the former is the main determinant of the unique aroma and important pharmacodynamic ingredient in agarwood[27–29]. Agarwood chips can cost from US$6,000 to US$20,000 per kilogram[30]. China has its own system for grading agarwood, which has started hundreds of years ago. Formerly, the classification of quality grades of agarwood in China was based on its sinking ability (i.e., the specific density of sinking in water, half floating or floating) and appearance characteristics (i.e., the sensory evaluation of aroma characteristics, color characteristics, and the resin area of agarwood). For the moment, whether it is commercial agarwood collected in the Forestry Industry Standard of the People's Republic of China (2017 edition), or medicinal agarwood collected in the Pharmacopoeia of the People's Republic of China (2020 edition), the quality of agarwood was evaluated by description (the characteristics of appearance, aroma, and taste), microscopic identification (microscopic characteristics of transverse section and interxylary phloem), physicochemical reaction identification of terpenoids, TLC identification, determination of the contents of ethanol-soluble extractives (EEC%) (mainly composed of sesquiterpenes and 2-(2-phenylethyl) chromones) and agaroretrol (one of the 2-(2-phenylethyl) chromones) content, and HPLC characteristic chromatogram (identification of 2-(2-phenylethyl) chromones). The above standard requires that EEC% not less than 10.0%, and the content of agaroretrol not less than 0.1%. Among them, EEC% is one of the important indexes of quality evaluation and quality classification of agarwood. Besides China, some Southeast Asian countries (e.g., Indonesia) also use this classification method. However, there is no uniform standard. Some regions in China (e.g., Dongguan and Hainan) divide agarwood into five quality grades based on EEC%, e.g., special Grade: extractives ≥ 30.0%; Grade I: 25.0% ≥ extractives > 30.0%; Grade II: 20.0% ≥ extractives > 25.0%, Grade III: 15.0% ≥ extractives > 20.0%, Grade IV: 10.0% ≥ extractives > 15.0%[31–33].

The aims of this paper are to evaluate the effects of different factors of complex inducer (comprehensive method) based on design of experiments (DoE) and to select an effective agarwood-inducing agent[34–36]. with a view to improving the agarwood-inducing efficiency and reducing the cost of agarwood-induction. Technology for artificial agarwood induction is mainly inspired by the natural agarwood formation process[37]. Nevertheless, the chemical composition and proportion of artificial agarwood are largely different from those of natural ones, and their quality and price are far less than those of natural agarwood[37–39]. For these reasons, it is of great importance to continuously improve the method and quality of artificial agarwood.

Results

Single factor experiment

Induction samples were collected for 5, 7 and 10 days, and GC–MS compared the total peak area of each induction sample (Table 1), and XCMS online platform was used to identify the common secondary metabolites of the samples induced by the same inducer (Table 2). Based on GC–MS analysis, the total peak area of the three induction formulations with different concentrations was 10% MeJA-D5 with the largest and 0.1% MeJA-D5 with the smallest. The results of the identification of different inducer groups, as shown in Table 2, showed that the common secondary metabolite in the MeJA group was MeJA, and the common secondary metabolite in the FA and A13 groups was benzaldehyde, 4-hydroxy-3,5-dimethoxy. For inducer containing 10% MeJA, although

No.	Sample	Total peak Area ($\times 10^8$)	No.	Sample	Total peak Area ($\times 10^8$)
S1	FA-0.05%-D5	1.96	S12	MeJA-10%-D5	51.2
S2	FA-0.5%-D5	1.10	S13	A13-5%-D5	1.70
S3	FA-1%-D5	1.44	S14	A13-10%-D5	2.80
S4	FA-0.05%-D7	0.46	S15	A13-15%-D5	3.18
S5	FA-0.5%-D7	0.46	S16	A13-5%-D7	2.78
S6	FA-1%-D7	0.41	S17	A13-10%-D7	0.92
S7	FA-0.05%-D10	1.10	S18	A13-15%-D7	1.03
S8	FA-0.5%-D10	1.11	S19	A13-5%-D10	2.87
S9	FA-1%-D10	1.12	S20	A13-10%-D10	3.26
S10	MeJA-0.1%-D5	0.40	S21	A13-15%-D10	2.84
S11	MeJA-1%-D5	0.99			

Table 1. Schedule and results from in vitro branch model of *A. sinensis* in single factor experiment.

No.	Inducers	Chemical Name	Formula	CAS	R.match	RI[a]	RI[b]
1	FA	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-○	$C_9H_{10}O_4$	134-96-3	916	1649.6	1627.6
2	MeJA	Methyl jasmonate▪	$C_{13}H_{20}O_3$	1211-29-6	954	1644	1608.9
3	A13	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-○	$C_9H_{10}O_4$	134-96-3	904	1649.6	1626.6

Table 2. Common secondary metabolites in single factor-induced samples identified by GC–MS. [a]Retention index value was retrieved from the NIST library. [b]The calculated value of retention index. ○ Aldehyde compounds, ▪ Ester compounds.

No.	Rt(min)	Chemical Name	Formula	CAS	R.match	RI[a]	RI[b]
1	20.488	2,4-Di-tert-butylphenol○	$C_{14}H_{22}O$	96-76-4	959	1512	1481.0
2	26.071	Methyl jasmonate▪	$C_{13}H_{20}O_3$	1211-29-6	968	1644	1608.0
3	34.831	Tetradecanoic acid☆	$C_{14}H_{28}O_2$	544-63-8	787	1798	1797.1
4	39.660	Hexadecanoic acid, methyl ester▪	$C_{17}H_{34}O_2$	112-39-0	853	1894	1879.7
5	42.011	*n*-Hexadecanoic acid☆	$C_{16}H_{32}O_2$	57-10-3	947	1929	1925.4

Table 3. Common secondary metabolites in complex inducer samples induced identified by GC–MS. [a]Retention index value was retrieved from the NIST library. [b]The calculated value of retention index. ○Phenolic compounds; ▪Ester compounds, ☆Fatty acid compounds.

the total peak area was the largest in the GC–MS analysis results, the preparation of MeJA inducers required a large amount of alcohol as a solvent, which would cause greater harm to the *A. sinensis*. Dripped with large amounts of ethanol, it can cause them to dehydrate, dry out, and then die.

Identification and screening of secondary metabolites

We used 16 combinations of MeJA (0.1%, 1%), FA (0.05%, 1%), A13 (5%, 15%) and time (7d, 15d) to treat the in vitro branches of *A. sinensis,* respectively. GC–MS analysis was performed on agarwood samples with different treatments, followed by chromatographic peak alignment using XCMS online and retrieval from the NIST 17 standard spectral library to identify common secondary metabolites in the samples (Table 3). The full spectrum data information was extracted through the XCMS online platform, and the components with difference in peak signal intensity of inducer treated samples for 7 and 15 days were screened based on OPLS-DA analysis. The arrangement experiment is an external model verification method to verify the fitting degree of OPLS-DA. By changing the arrangement order of the categorical variable y randomly multiple times (n = 200), various random Q^2 values were produced. The results indicate that the model is effective and there is no overfitting phenomenon (Fig. 1B). According to the OPLS-DA scatter plots (R^2X: 0.936, R^2Y: 0.962, and Q^2: 0.756) (Fig. 1A), samples with different processing times can be clearly distinguished. Based on the corresponding VIP values (Fig. 1C), the variable with VIP value > 1 was selected, and the result showed that the component with difference between the two types of samples was MeJA.

2^4 factorial design experiment

Model fitting and ANOVA analysis

The 2^4 factorial design was carried out using Design-Expert software (Version 8.0.6.1). Sixteen design experiments were performed to find the optimal complex inducer formulation by varying the concentrations of MeJA (A), FA (B), and A13 (C) and time (D).

Their lower and higher levels were designated as -1 and 1, respectively. The relative content of MeJA (%, Y_1) and total peak area ($\times 10^8$, Y_2) were used as response values. The Y_1 and Y_2 were normalised, and the geometric mean of normalised values of each index was calculated to obtain the total normalised value (*OD*, maximum) (Table 4).

Therefore, a 2^4 factorial design was employed to study the impact of the four factors, and a total of 16 runs were performed (Table 5). The results obtained were statistically evaluated using Design-Expert software (Version 8.0.6.1).

The Model *F*-value of 21.58 implies the model is significant. There is only a 0.06% chance that a "Model *F*-Value" this large could occur due to noise (Table 6). Values of "Prob > *F*" less than 0.0500 indicate model terms are significant. In this case A, B, D, AB, AD, BD, ABC, BCD are significant model terms. This means that the main effects of A, B, D and the interactions of AB, AD, BD, ABC, BCD have significant effects on the response variable -*OD* values.

In addition, the determination coefficient R^2 value was 0.9805, the Adj. R^2 value was 0.9351 and the Adeq. Precision value was 16.667 (> 4.0). These values indicate that the regression model has a high fitting degree and low error value. Additionally, it accurately reflects the actual change rule of the experimental group. The R^2 value showed that 98.05% of the test data could be explained by the regression model. The R^2 value was close to the Adj. R^2 value and approached 1. This reflects that the high fitting degree of the factors to the total normalised value (*OD*). The *CV* value of 26.6% (> 15%) indicated that the response index varied greatly among different samples, suggesting that the induction effects of samples were different to a certain extent.

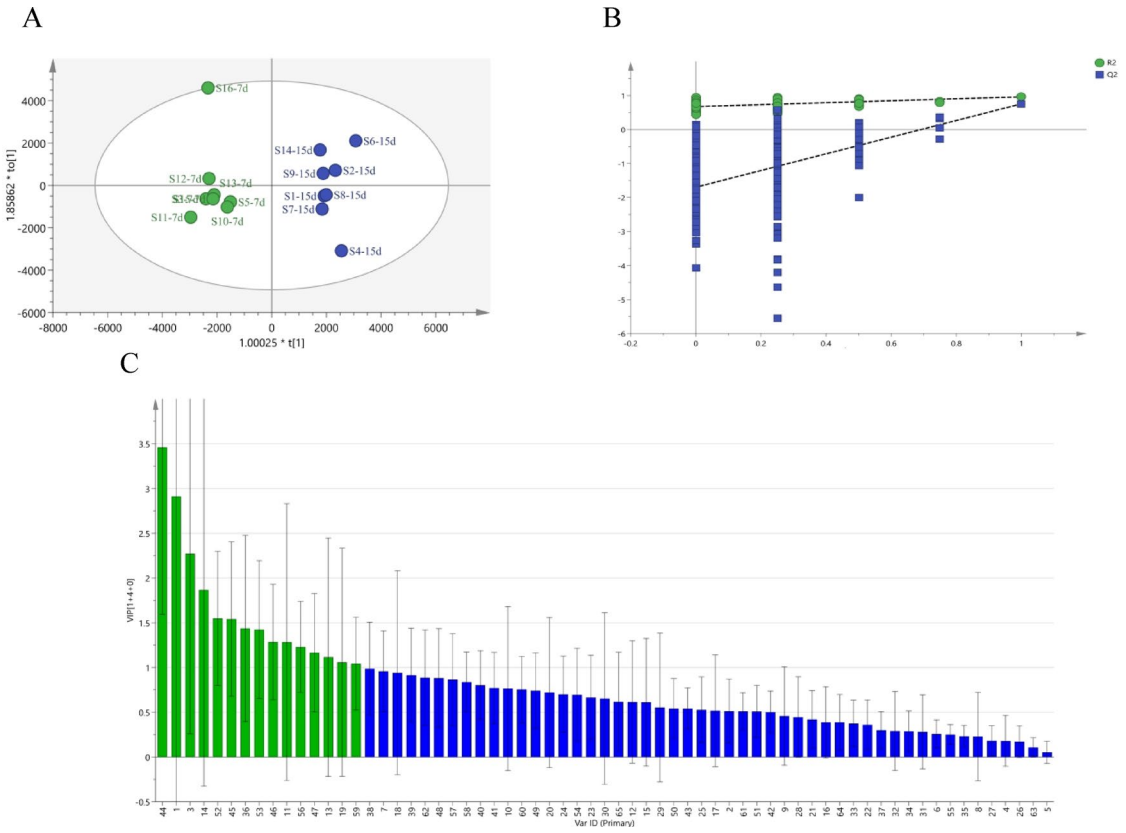

Fig. 1. (**A**) Scatter plot, (**B**) Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) arranges the model verification diagram, and (**C**) Variable Importance in Projection (VIP) scores of peak signal intensities were compared between samples treated with inducers for 7 and 15 days. VIP values exceeding 1 are in green.

Factors	Levels	
	Low (− 1)	High (+ 1)
A-MeJA concentration (%)	0.1	1
B-FA concentration (%)	0.05	1
C-A13 concentration (%)	5	15
D-Time (day)	7	15
Responses	Constraint	
Y_1-Relative content of MeJA (%)	Maximum	
Y_2- Total peak Area ($\times 10^8$)	Maximum	
OD	$d_{max} = (Y_i - Y_{min})/(Y_{max} - Y_{min})$(2)	
	$OD = (d_1 + d_2 + \cdots + d_k)/k$(3)	

Table 4. 2^4 factorial design factors and their levels. [1]In Eq. (2), d_{max} is the normalised value of each response value, Y_{min} is the minimum value of each response value, and Y_{max} is the maximum value of each response value. In Eq. (3), *OD* is the total normalised value, and *k* is the number of indicators.

Runs	Coded variables				Responses		
	A-MeJA	B-FA	C-A13	D-Time	Y_1	Y_2	*OD*
1	−1	1	−1	1	0.31	1.74	0.000
2	1	−1	−1	1	1.04	2.8	0.088
3	1	1	1	−1	24.66	6.2	0.643
4	−1	−1	−1	1	0.78	5.31	0.270
5	−1	1	−1	−1	7.59	3.09	0.193
6	1	−1	1	1	1.23	3.58	0.148
7	−1	−1	1	1	0.49	2.53	0.061
8	−1	1	1	1	0.65	2.7	0.075
9	1	1	−1	1	1.57	2.4	0.065
10	1	−1	−1	−1	2.13	3.88	0.182
11	−1	−1	1	−1	6.05	3.38	0.195
12	1	1	−1	−1	39.28	8.49	1.000
13	1	−1	1	−1	4.37	7.6	0.486
14	1	1	1	1	8.18	3.19	0.208
15	−1	−1	−1	−1	9.73	2.62	0.186
16	−1	1	1	−1	5.65	6.18	0.397

Table 5. 2^4 Factorial design runs.

Source	Sum of Squares	df	Mean Square	*F*-Value	*P*-Value	
Model	1.6000	14	0.1100	21.58	0.0006	Significant
A-MeJA	0.1700	1	0.1700	32.52	0.0013	
B-FA	0.0660	1	0.0660	12.44	0.0124	
C-A13	0.0031	1	0.0031	0.59	0.4711	
D-Time	0.4100	1	0.4100	76.98	0.0001	
AB	0.0790	1	0.0790	15.00	0.0082	
AC	0.0002	1	0.0002	0.03	0.8647	
AD	0.1300	1	0.1300	24.30	0.0026	
BC	0.0000	1	0.0000	0.01	0.9262	
BD	0.1600	1	0.1600	30.88	0.0014	
CD	0.0003	1	0.0003	0.06	0.8114	
ABC	0.0870	1	0.0870	16.44	0.0067	
ABD	0.0220	1	0.0220	4.21	0.0861	
ACD	0.0290	1	0.0290	5.53	0.0569	
BCD	0.0550	1	0.0550	10.35	0.0182	
Residual	0.0320	6	0.0053			
Lack of Fit	0.0320	1	0.0320			
Pure Error	0	5	0			
Cor Total	1.63	20				

Table 6. Analysis of variance (ANOVA) of data from 2^4 factorial design tests. 1 C.V. = 26.6%; $R^2 = 0.9805$; Adj $R^2 = 0.9351$; Adeq. precision = 16.667.

After analysing and calculating each factor and interaction between the effect of the response value by Design Expert 8.0.6.1 software, an equation for the data were obtained as follows:

$$\begin{aligned} OD = {} & 0.26 + 0.095\mathrm{A} + 0.059\mathrm{B} + 0.013\mathrm{C} - 0.15\mathrm{D} + 0.065\mathrm{AB} \\ & + 0.002968\mathrm{AC} - 0.082\mathrm{AD} - 0.001603\mathrm{BC} - 0.092\mathrm{BD} - 0.00416\mathrm{CD} \\ & - 0.068\mathrm{ABC} - 0.034\mathrm{ABD} + 0.039\mathrm{ACD} + 0.054\mathrm{BCD} \end{aligned} \quad (1)$$

where *OD* represents the response, and A, B, C and D are the mathematical model terms for the four factors, including MeJA concentration, FA concentration, A13 concentration and time, respectively. The correlation coefficient of the Eq. (1) shows that the model fitted well and can be used to analyze and predict the screening of the complex inducer.

In Eq. (1), the mean value of the total normalised value (*OD*) was 0.26 for 2^k factorial test. For each factor, the most important factor influencing the *OD* values was induction time, and its effect estimation was −0.15,

an indication of a negative effect on the *OD* value. The second most important factor was the concentration of MeJA, and its effect estimation was + 0.095, an indication of a positive effect on the *OD* value. However, formic acid had less influence on the *OD* value; its effect estimation was + 0.059, which is indicative of a positive effect on the *OD* value. For interaction factors with influential effects, the factors AB, AC, ACD, BCD had a positive effect on the *OD* value, whereas the factors AD, BC, BD, CD, ABC and ABD had a negative effect.

Pareto analysis

To compare the contribution ratio of each single factor and interaction to the *OD* value, pareto analysis method, was used (Fig. 2).

The contribution ratios between the main influencing factors in descending order was D > A > B > C. Then, the contribution ratio of two-factor interaction in descending order was BD > AD > AB > CD > AC > BC. The contribution ratios of the multiple interacting factors in descending order was ABC > BCD > ACD > ABD (Fig. 2.). The calculation showed that the total contribution percentage of A (13.92%), B (5.37%), and D (34.70%) was more than 50%, while that of C (0.26%) was only 0.30%. Therefore, the effect of A-MeJA and B-FA concentrations and D-Time on the *OD* value was significant, as calculated from the relative content of MeJA and total peak area. The influence of C-A13 inducer at a concentration range of 5–15% on the *OD* value was low; however, it was a positive influence.

In the table showing the results from 2^k factorial design optimisation using Design Expert 8.0.6, the first row is the optimised complex inducer subschema (Table 7). Comprehensive analysis of the above ANOVA analysis and Pareto analysis, the complex inducer was found to be "1% MeJA + 1% FA + A13" and this complex inducer was used in the field experiment.

Verification of factorial experimental results

In this study, we used in vitro branches of *A. sinensis* as experimental materials and evaluated the induction effects of various single inducers and complex inducers. The relative content of MeJA and the total peak area of the samples were employed as assessment indicators. The induction results were evaluated using two algorithms: TOPSIS and the calculation of *OD* values (Table 8). Both algorithms consistently demonstrated that the combination of 1% MeJA + 1% FA + A13 yielded the best induction effect, which is in agreement with the screening results from the factorial experiment. For the single inducer MeJA, a concentration of 10% MeJA was found to cause dehydration and death of the tree, as also mentioned in this paper. Unfortunately, after 7 days of induction with MeJA, we were unable to obtain experimental data from the sample due to an experimental error. Nevertheless, based on the2^k factorial screening results and extensive in vitro branch experiments, we have confirmed that the most effective inducer is the combination of 1% MeJA + 1% FA + A13.

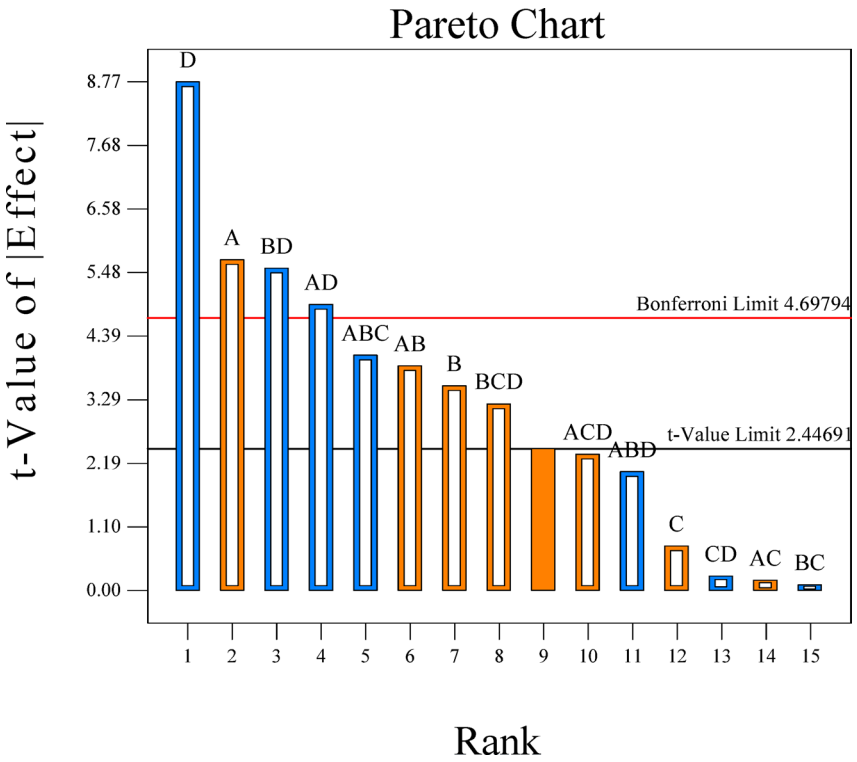

Fig. 2. Graphical visualisation of pareto analysis of complex inducer factors.

Number	A-MeJA	B-FA	C-A13	D-Time	*OD*	Desirability	
1	1	1	5	7	0.9758	0.976	Selected
2	1	1	15	7	0.6913	0.691	
3	1	0.05	15	7	0.4377	0.438	
4	0.1	1	15	7	0.3490	0.349	
5	0.1	0.05	5	15	0.3190	0.319	
6	1	0.05	5	7	0.2303	0.230	
7	0.1	0.05	15	7	0.2194	0.219	
8	0.1	1	5	7	0.2176	0.218	
9	1	0.05	15	15	0.1966	0.197	
10	1	1	15	15	0.1599	0.160	
11	0.1	0.05	5	7	0.1376	0.138	
12	0.1	1	15	15	0.1239	0.124	
13	1	1	5	15	0.0893	0.089	
14	1	0.05	5	15	0.0636	0.064	
15	0.1	0.05	15	15	0.0124	0.012	

Table 7. Best complex inducer factors.

TOPSIS evaluation calculations			*OD* value calculations		
Inducers	Relative proximity C	Rank	Inducers	*OD*	Rank
10%MeJA-D10	1.000	1	1%MeJA + 1%FA + 5%A13-D7	0.785	1
1%MeJA + 1%FA + 5%A13-D7	0.430	2	10%MeJA-D10	0.644	2
1%MeJA + 1%FA + 10%A13-D7	0.313	3	1%MeJA + 1%FA + 10%A13-D7	0.586	3
1%MeJA + 1%FA + 15%A13-D7	0.286	4	1%MeJA + 1%FA + 15%A13-D7	0.541	4
1%MeJA + 0.05%FA + 15%A13-D7	0.197	5	1%MeJA + 0.05%FA + 15%A13-D7	0.470	5
0.1%MeJA + 1%FA + 15%A13-D7	0.167	6	0.1%MeJA + 1%FA + 15%A13-D7	0.395	6
1%MeJA + 0.05%FA + 10%A13-D7	0.145	7	1%MeJA + 0.05%FA + 10%A13-D7	0.298	7
1%MeJA-D10	0.120	8	0.1%MeJA + 1%FA + 5%A13-D7	0.228	8
0.1%MeJA + 0.05%FA + 5%A13-D7	0.111	9	0.1%MeJA + 0.05%FA + 15%A13-D7	0.215	9
0.1%MeJA + 0.05%FA + 10%A13-D7	0.106	10	0.1%MeJA + 1%FA + 10%A13-D7	0.209	10
0.1%MeJA + 1%FA + 5%A13-D7	0.103	11	0.1%MeJA + 0.05%FA + 10%A13-D7	0.204	11
0.1%MeJA + 1%FA + 10%A13-D7	0.099	12	0.1%MeJA + 0.05%FA + 5%A13-D7	0.199	12
0.1%MeJA + 0.05%FA + 15%A13-D7	0.098	13	1%MeJA + 0.05%FA + 5%A13-D7	0.198	13
1%MeJA + 0.05%FA + 5%A13-D7	0.084	14	1%MeJA-D10	0.193	14
1%MeJA + 15%A13-D7	0.077	15	1%MeJA + 15%A13-D7	0.181	15
5%A13-D7	0.064	16	5%A13-D7	0.144	16
1%MeJA + 10%A13-D7	0.057	17	1%MeJA + 10%A13-D7	0.130	17
0.1%MeJA-D10	0.048	18	0.1%MeJA-D10	0.090	18
1%MeJA + 5%A13-D7	0.032	19	1%MeJA + 5%A13-D7	0.075	19
0.1%MeJA + 15%A13-D7	0.021	20	0.1%MeJA + 15%A13-D7	0.041	20
15%A13-D7	0.018	21	15%A13-D7	0.039	21
0.1%MeJA + 10%A13-D7	0.017	22	10%A13-D7	0.033	22
10%A13-D7	0.015	23	0.1%MeJA + 10%A13-D7	0.032	23
0.05%FA + 10%A13-D7	0.006	24	0.05%FA + 10%A13-D7	0.013	24
0.1%MeJA + 5%A13-D7	0.004	25	0.1%MeJA + 5%A13-D7	0.010	25
0.5%FA-D7	0.002	26	0.05%FA-D7	0.005	26
0.05%FA + 0.1%A13-D7	0.002	27	0.5%FA-D7	0.005	26
0.05%FA-D7	0.002	28	0.05%FA + 0.1%A13-D7	0.005	26
1%FA-D7	0.001	29	1%FA-D7	0.002	29
0.05%FA + 1%A13-D7	0.000	30	0.05%FA + 1%A13-D7	0.000	30

Table 8. Evaluation of inducer induction effect by two algorithms.

No.	Group	Inducers	Sample Tag	Resin formation time	Date of collection	Growth conditions
1	Experimental I	1% MeJA + 1% FA + A13	F1-1	4 months	3–3-2022	Good growth condition but less leaves
			F1-2			Good growth condition but less leaves
			F1-3			Good growth condition but less leaves
2	Negative control I	25% ethanol	F2-1			Good growth condition, few leaves
			F2-2			Bad growth condition, leaves completely dropped
			F2-3			Good growth condition, few leaves
3	Blank control I	H_2O	F3-1			Good growth condition, dense leaves
			F3-2			Good growth condition, dense leaves
			F3-3			Good growth condition, dense leaves
4	Experimental II	1% MeJA + 1% FA + A13	N1-1	9 months	24–7-2022	Good growth condition but less leaves
			N1-2			Good growth condition but less leaves
			N1-3			Good growth condition but less leaves
5	Negative control II	25% ethanol	N2-1			Good growth condition, few leaves
			N2-2			Good growth condition, few leaves
			N2-3			Good growth condition, few leaves
6	Blank control II	H_2O	N3-1			Good growth condition, dense leaves
			N3-2			Good growth condition, dense leaves
			N3-3			Good growth condition, dense leaves

Table 9. Collection information of *A. sinensis* samples.

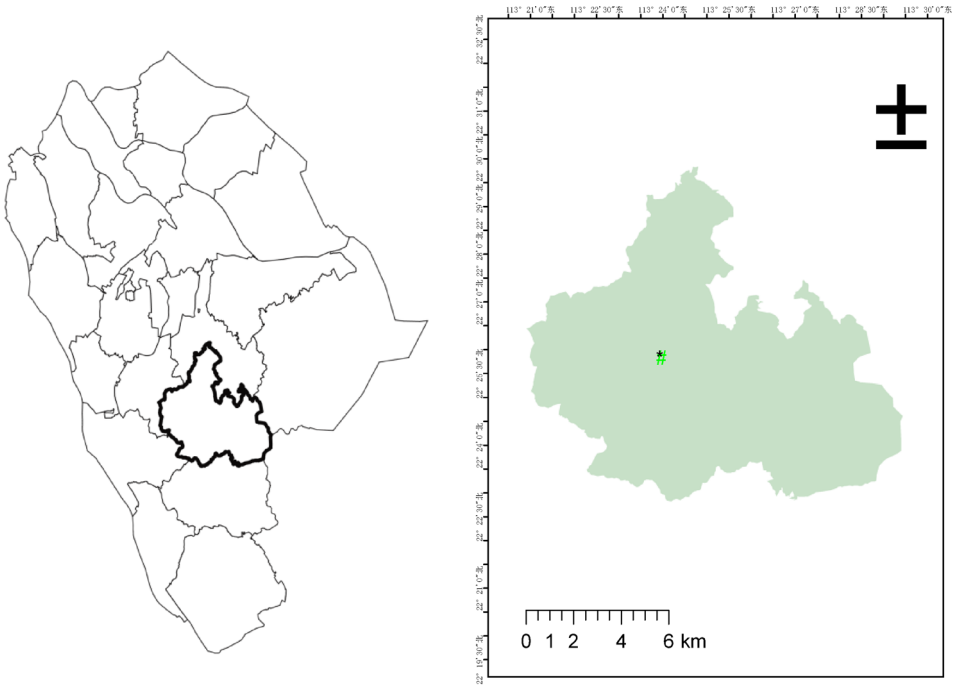

Fig. 3. Geographical map of agarwood sample resin collection area.

Sample collection in field experiment

Due to the complex environmental factors in the field experiment, the growth conditions of *A. sinensis* trees was different. Three parallel samples were prepared for each group. For the wood sample collection, the *A. sinensis* of four months (F1-1–F1-3, F2-1–F2-3, F3-1–F3-3) and nine months (N1-1–N1-3, N2-1–N2-3, N3-1–N3-3) in the field experiment were cut down, and the resinous heartwood were shaved and used as test items. A total of 18 batches of *A. sinensis* wood samples were collected, and the detailed information is presented in Table 9. The geographic map of the sampling area is shown in Fig. 3.

The growth condition of the experimental *A. sinensis* was good. The area of incense part of the experimental group samples was bigger than that of the control group, and comparing the 9-month samples with the 4-month

samples, the brownish-black resin part was more thickened. In the second group, in which 25% ethanol was used as the negative control solvent, the *A. sinensis* trees were largely damaged and in turn had fewer leaves and rottenly xylem. In the third group, in which distilled water was used as the blank control, the *A. sinensis* trees grew well and could be used for subsequent incense making process (Fig. 4).

Analysis of quality evaluation

Moisture content, EEC%, and colour reaction

As shown in Table 10, the moisture contents of the experimental group, negative control group, and blank group were 4.35–5.94%, 4.09–6.29%, and 5.75–6.17%, respectively. EEC% of the experimental group, negative control group, and blank control group were 4.63–14.80%, 6.90–10.85%, and 3.51–5.03%, respectively. The colour reaction of the 4-month wood samples did not conform to ChP 2020, but that of the 9-month samples of the experimental group was cherry red, which fits with ChP 2020.

Content of agarotetrol

Solutions of 18 batches of wood samples, one batch of agarwood reference drugs (Sample tag: DZYC), and agarotetrol reference substance were analyzed by HPLC. The contents of agarotetrol of the experimental group, negative control group, and blank group were 0.02–0.86%, 0.09–0.18%, and 0.01–0.05%, respectively. The content of agarotetrol in DZYC was 0.38% (Table 10).

Analysis of fingerprint and chromatogram

HPLC chromatogram

Test solutions of 18 batches of wood samples and two batches of DZYC were subjected to HPLC analysis. The samples from both the experimental group and negative control group showed the six characteristic peaks of ChP 2020 and agarwood reference drug. Using the chromatogram of DZYC as the reference, the similarity of the experimental group, negative control group, and blank control group was found to be 0.885–0.960, 0.694–0.835, and 0.039–0.638, respectively (Table 10). The overlapped HPLC chromatogram of 18 batches of samples is shown in Fig. 5.

Chromatographic fingerprint analysis by GC–MS

Chromatographic fingerprint can be used to evaluate the similarity among samples and obtained by calculating the correlation coefficient of the original data. The test sample solutions were prepared and analyzed by GC–MS.

The GC–MS analysis results for each sample were normalised by the peak area, and the obtained data files were then imported into the Traditional Chinese Medicine Fingerprint (2012 version) software. Similarity between a set of chromatographic fingerprints of the samples and the agarwood reference drug was evaluated and calculated. The GC–MS chromatographic fingerprint of the samples was established (Fig. 6). Obtaining the total ion chromatogram (TIC) of a sample took a long period of time; thus, after automatic matching with multi-point correction in Traditional Chinese Medicine Fingerprint (2012 version) software, three characteristic peaks of three compounds, including benzyl acetone (Rt = 12.352 min), baimuxinal (Rt = 58.547 min) and 2-(2-phenylethyl) chromone (Rt = 140.734 min), were used as the reference peaks in subsequently matching. Due to their good separation and high response values, they are suitable to be used as the reference peaks (these peaks were marked in Fig. 6a as TIC of DZYC). With the control fingerprint as the reference, the similarity for the experimental group, negative control group, and blank control group were found to be 0.640–0.943, 0.659–0.911, and 0.019–0.205, respectively (Table 10). In addition, the size of total peak area of the three groups could be ranked as follows: experimental group > negative control group > blank group. It could be seen that the experimental group and the blank group were obviously different (Fig. 6b, c, and d).

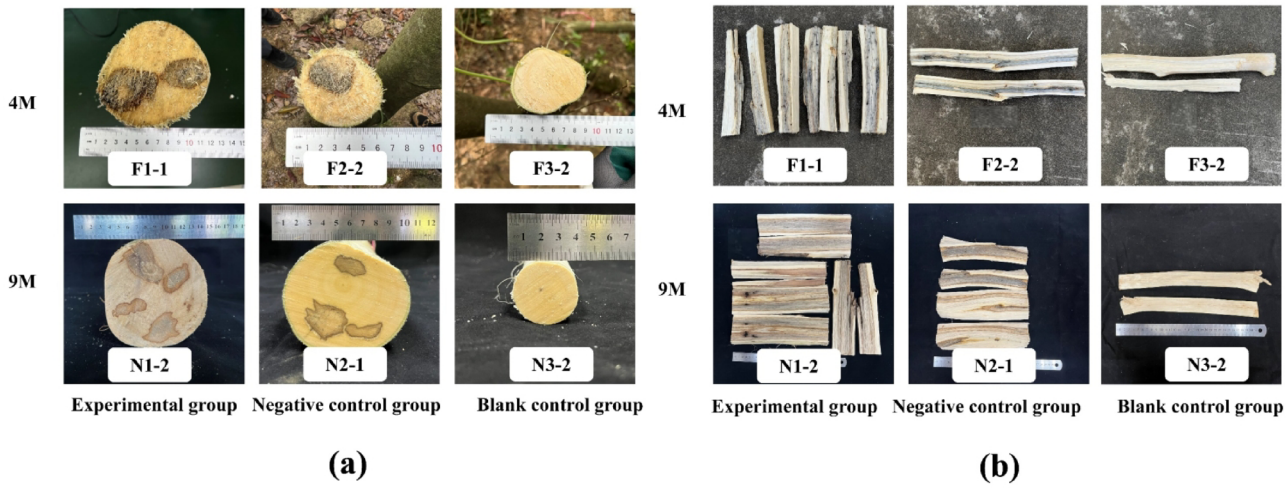

Fig. 4. Comparation of transverse (**a**) and longitudinal (**b**) sections of 4 M and 9 M *A. sinensis* wood samples.

Group	No.	Sample tag	GC–MS fingerprint chromatogram			HPLC characteristic fingerprint chromatogram	Quality evaluation			
			Similarity	R^a	Total peak area ($\times 10^8$)	Similarity	Moisture (%)	EEC (%)	Colour-reaction value assignment[b]	Agarotetrol (%)
Experimental	1	F1-1	0.811	1.42	25.6	0.931	5.78	6.08	0	0.23
	2	F1-2	0.806	1.6	15.1	0.896	5.94	4.63	0	0.02
	3	F1-3	0.64	4.76	10.7	0.903	5.12	14.77	0	0.27
	4	N1-1	0.934	2.33	44.8	0.885	5.29	12.83	2	0.86
	5	N1-2	0.903	1.63	67.3	0.96	4.35	13.81	3	0.19
	6	N1-3	0.943	1.92	13	0.941	4.72	15.97	3	0.33
Negative control	7	F2-1	0.663	1.64	6.07	0.816	6.21	7.5	0	0.1
	8	F2-2	0.708	1.32	6.97	0.694	6.29	7.22	0	0.1
	9	F2-3	0.757	1.37	6.54	0.805	6.19	6.9	0	0.09
	10	N2-1	0.884	0.8	13.5	0.835	4.09	10.19	1	0.16
	11	N2-2	0.659	0.71	36	0.76	4.82	10.85	1	0.15
	12	N2-3	0.911	1.2	10.5	0.814	5.26	9.52	1	0.18
Blank control	13	F3-1	0.049	4.3	1	0.039	6.59	5.03	0	0.01
	14	F3-2	0.06	2.6	1.05	0.127	5.75	4.74	0	0.05
	15	F3-3	0.205	3.67	1.21	0.046	6.14	3.51	0	0.02
	16	N3-1	0.019	4.32	2.53	0.638	5.93	3.95	0	0.02
	17	N3-2	0.022	4.98	0.57	0.073	6.17	4.58	0	0.02
	18	N3-3	0.096	5.8	1.13	0.063	6.13	4.37	0	0.02
Reference	19	[c]DZYC	1	1.05	33.1	1	–	–	–	0.38

Table 10. Results on similarity and apparent abundance (R) of GC–MS fingerprint chromatogram, HPLC characteristic fingerprint chromatogram, and quality evaluation of *A. sinensis* wood samples and agarwood reference drug. [a]R (apparent abundance) = $A_{139–220\ min}/A_{0–139\ min}$. The ratio of sum peak area in the range of 139–220 min range of individual agarwood and that in the range of 0–139 min. [b]Colour reaction value assignment: Purple = 4, dark cherry red = 3, cherry red = 2, pale cherry red = 1, other colour = 0. [c]DZYC: agarwood reference drug.

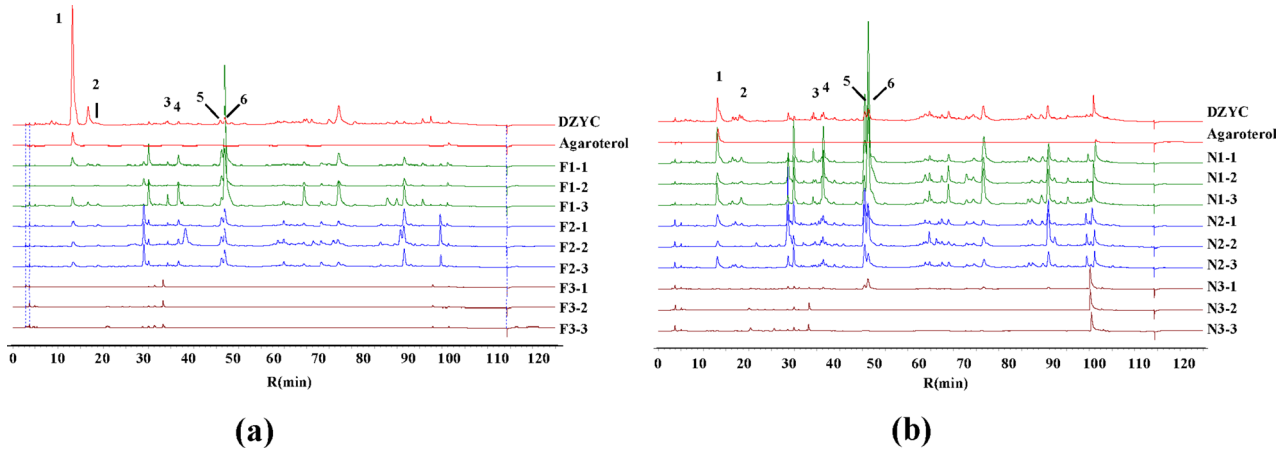

Fig. 5. Overlapped HPLC chromatogram for *A.* sinensis wood samples and reference: (**a**) 9 batches of 4 M samples; and (**b**) 9 batches of 9 M samples. [1]In the six characteristic peaks, peak 1: agarotetrol; peak 3: 8-chloro-2-(2-phenylethyl)-5,6,7-trihydroxy-5,6,7,8-tetrahydrochromone; and peak 5: 6,4′-dihydroxy-3'-methoxy-2-(2-phenylethyl) chromone. [2]DZYC: agarwood reference drug.

The ratio (R) of the sum peak area at the 139–220 min range of individual agarwood to that at the 0–139 min range was determined and used as the apparent abundance of GC–MS fingerprints. The ratios of the experimental group, negative control group, and blank control group were found to be 1.42–4.76, 0.80–1.64, and 2.60–5.80, respectively (Table 10), and the ratio of DZYC was 1.05. In addition, the secondary metabolites in the agarwood samples in the experimental group were identified (Table 11).

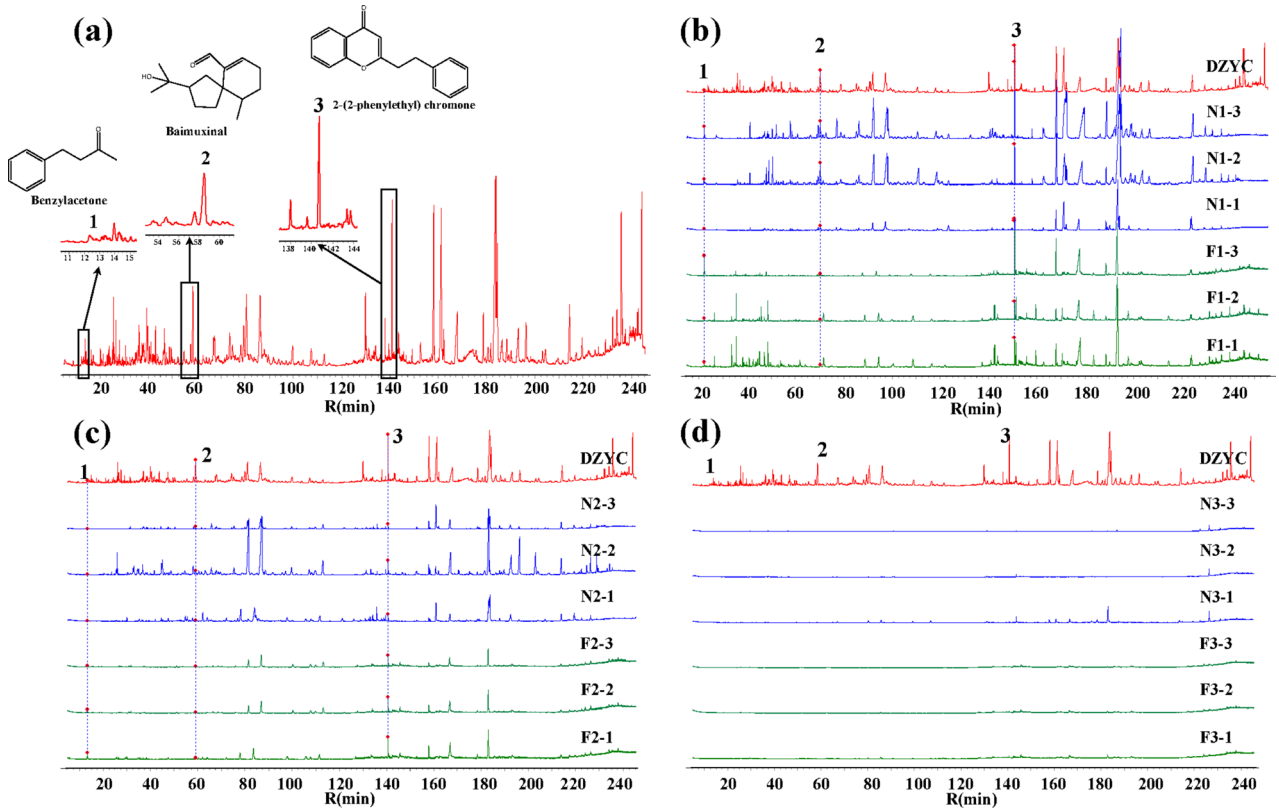

Fig. 6. GC–MS fingerprint of (**a**) agarwood reference drug and (**b**)–(**d**) overlap of fingerprint for 6 batches of experimental group (**b**), negative control group (**c**), and blank control group (**d**) and that for 1 batch of agarwood reference drug. Peak 1: benzylacetone; peak 2: baimuxinal; and peak 3: 2-(2-phenylethyl) chromone.

Correlation between EEC% and total HPLC total peak area and agarwood formation time

Multiple comparison tests on EEC (%) and total HPLC peak area ($\times 10^4$) of artificial agarwood samples induced by complex inducer for 4 and 9 months were performed using GraphPad Prism 8.4.0. Compared to the control group, EEC% and total HPLC peak area ($\times 10^4$) of the experimental group significantly increased with increasing induction time ($P < 0.05$, Fig. 7). This indicates that the induction by complex inducer could lead to the accumulation of 2-(2-phenylethyl) chromones.

Discussion

Up to now, a variety of artificial inducer have been used to induce agarwood formation in *A. sinensis*. MeJA, as a plant hormone, plays an important role in the formation of agarwood. The content of agarotetrol reached 1.78 mg/g, which was 14.8–21.8 times higher than that of the control group after induction of *A. sinensis* with exogenous MeJA (10 g/L)[10]. Injecting appropriate amounts of acidic or alkaline inducing agents, such as salicylic acid, ethylene glycol, formic acid, and sodium dihydrogen phosphate, into the xylem of *A. sinensis* can promote the production of resin, with the EEC% reaching over 10.0%, and the highest reaching up to 32.4%, in compliance with the pharmacopeial standards for medicinal use in the Chinese Pharmacopoeia[40]. After one year of induction treatment with sulfuric acid and sodium methylbisulfate, the content of agarwood oil produced by *A. crassna* was 0.038–0.039%, and the sesquiterpenes accounted for 15.80–20.80% of the total oil[41]. *B. rhodina is* a plant pathogenic fungus, which can infect more than 500 kinds of tropical and subtropical forests[42] and cause a variety of plant diseases[43]. *B. rhodina* A13 is an endophytic fungus isolated from the resinous xylem of *A. sinensis*, can induce *A. sinensis* excised twigs to produce 5,9-dimethyl-2-(1-methylethylidene) cyclodecanol, sesquiterpene of agarwood[44]. Using sawdust of *A. sinensis* as the culture substrate, solid fermentation culture was performed on *B. rhodina* A13. Seven 2-(2-phenylethyl) chromone analogues and ten steroid compounds, as well as flavonoid compounds, were isolated from *B. rhodina* A13. Among these, the steroid compounds exhibited good cytotoxic activity[45]. After 12 months of induction with FA + *B. rhodina* A13 in *A. sinensis*, EEC% of agarwood could reach a maximum of 40.60%, with a median value of 15.54% across 45 batches[46]. Therefore, we selected MeJA, FA and A13 as candidate inducers. Based on the survival rate of trees and continuous testing[39,46], the appropriate concentration is obtained for single-factor experiments. Because the in vitro branches of *A. sinensis* were induced for a short time and did not produce large amounts of sesquiterpenes and 2-(2-phenylethyl) chromones, we assessed the degree of stress by changes in the accumulation of secondary metabolites. Therefore, the total peak area of the sample is used as the evaluation index. Based on GC–MS analysis, in the MeJA group, the total peak area of samples induced by 10%MeJA was the largest, but the preparation of MeJA inducer required a large

No.	Rt (min)	Compound	Formula	CAS	RI[a]	RI[b]	Relative percentage (%)		
							N1-1	N1-2	N1-3
1	12.352	2-Butanone, 4-phenyl-○	$C_{10}H_{12}O$	2550–26-7	1245	1293.3	0.232	0.323	0.492
2	17.107	Hydrocinnamic acid○	$C_9H_{10}O_2$	501–52-0	1361	1366.9	0.25	0.082	0.228
3	19.245	Benzenepropanal, 2-oxo-○	$C_9H_8O_2$	56,485–04-2	1398.5	1400.5	–	0.018	–
4	25.641	Benzeneacetic acid, 4-methoxy-○	$C_9H_{10}O_3$	104–01-8	1496	1499.3	–	0.218	–
5	26.669	2H-1-Benzopyran-2-one, 6-methyl-○	$C_{10}H_8O_2$	92–48-8	1560.5	1515.6	–	0.08	0.033
6	28.482	Cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-, [1R-(1α,3α,4β)]- ▪	$C_{15}H_{26}O$	639–99-6	1557.1	1543.4	–	0.038	0.023
7	30.499	(-)-Spathulenol ▪	$C_{15}H_{24}O$	77,171–55-2	1582	1574.6	–	–	0.409
8	30.504	Caryophyllene oxide ▪	$C_{15}H_{24}O$	1139–30-6	1593	1574.6	0.375	0.419	–
9	35.438	10-epi-γ-Eudesmol ▪	$C_{15}H_{26}O$	15,051–81-7	1621	1627.8	–	–	0.053
10	35.463	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-α,α,4a,8-tetramethyl-, (2R-cis)- ▪	$C_{15}H_{26}O$	1209–71-8	1633	1634.9	–	0.049	–
11	35.892	β-Eudesmol▪	$C_{15}H_{26}O$	473–15-4	1639	1639.3	0.285	–	–
12	36.035	2-((2R,8R,8aS)-8,8a-Dimethyl-1,2,3,4,6,7,8,8a-octahydronaphthalen-2-yl)propan-2-ol ▪	$C_{15}H_{26}O$	20,489–45-6	1661	1642.5	–	0.155	–
13	36.342	Agarospirol ▪	$C_{15}H_{26}O$	1460–73-7	1642.1	1643.3	0.572	–	0.399
14	37.538	α-Cadinol ▪	$C_{15}H_{26}O$	481–34-5	1653	1656.1	0.299	–	–
15	38.029	2-((2R,4aR,8aS)-4a-Methyl-8-methylenedecahydronaphthalen-2-yl)acrylaldehyde ▪	$C_{15}H_{22}O$	3650–40-6	1691.4	1662.0	–	1.239	–
16	39.458	2-((2R,4aR,8aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphthalen-2-yl)acrylaldehyde▪	$C_{15}H_{22}O$	4586–01-0	1695.4	1675.8	–	0.217	0.405
17	41.099	(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol ▪	$C_{15}H_{24}O$	81,968–62-9	1694.5	1693.5	–	–	0.582
18	46.587	Ethyl 2,4-dihydroxy-6-methylbenzoate○	$C_{10}H_{12}O_4$	2524–37-0	1743.2	1725.8	–	0.25	–
19	47.35	Isolongifolol▪	$C_{15}H_{26}O$	1139–17-9	1781.2	1740.7	–	–	0.352
20	58.547	Baimuxinal ▪	$C_{15}H_{24}O_2$	86,408–21-1	–	1789.2	1.295	1.687	1.04
21	81.164	Bohlmann k2631 ▪	$C_{15}H_{20}O_2$	80,367–94-8	1916	1910.1	3.041	5.22	4.266
22	86.751	(3aR,4aS,5R,9aS)-5,8-Dimethyl-3-methylene-3a,4,4a,5,6,7,9,9a-octahydroazuleno[6,5-b] furan-2(3H)-one▪	$C_{15}H_{20}O_2$	–	1948	1952.0	–	0.225	–
23	140.734	2-(2-phenylethyl)chromone▲	$C_{17}H_{14}O_2$	61,828–53-3	–	2294.7	1.381	1.42	2.324
24	143.926	6-hydroxy-2-(2-phenylethyl)chromone▲	$C_{17}H_{14}O_3$	84,294–90-6	–	–	–	0.043	0.057
25	147.931	6-hydroxy-2-(2-phenylethyl)chromone▲	$C_{17}H_{14}O_3$	84,294–90-6	–	2417.0	0.255	0.298	0.299
26	152.653	5,8-dihydroxy-2-(2-phenylethyl)chromone▲	$C_{17}H_{14}O_4$	69,809–24-1	–	2596.7	–	1.45	0.428
27	153.048	6-hydroxy-2-(2-phenylethyl)chromone▲	$C_{17}H_{14}O_3$	84,294–90-6	–	–	–		0.786
28	158.153	6-methoxy-2-phenethyl-4H-chromen-4-one▲	$C_{18}H_{16}O_3$	84,294–89-3	–	2509.3	4.158	4.606	5.735
29	158.44	A methoxy group as the B-ring of 2-(2-phenylethyl)chromone▲	.	–	–	2670.7	–	0.382	–
30	162.256	5,8-dihydroxy-2-(2-phenylethyl)chromone▲	$C_{17}H_{14}O_4$	69,809–24-1	–	–	10.963	–	9.783
31	169.658	A hydroxy group as the A-ring of 2-(2-phenylethyl)chromone▲	.	–	–	2802.6	5.089	6.295	6.531
32	178.915	6-methoxy-2-(3-methoxyphenethyl)-4H-chromen-4-one▲	$C_{19}H_{18}O_4$	84,294–88-2	–	2900.0	2.424	1.406	2.605
33	184.686	5,8-dihydroxy-2-(2-(4-methoxyphenyl)ethyl)chromone▲	$C_{18}H_{16}O_5$	128,922–70-3	–	2911.5	24.585	19.048	20.76
34	185.021	5,8-dihydroxy-2-(2-(5-methoxyphenyl)ethyl)chromone▲	$C_{18}H_{16}O_5$	128,922–70-3	–	–	2.761	2.605	1.359
35	189.285	6-hydroxy-7-methoxy-2-(2-phenylethyl)chromone▲	$C_{18}H_{16}O_4$	–	–	2923.4	–	–	2.018
36	193.554	6-hydroxy-2-(2-(4'-methoxyphenyl)ethyl)chromone▲	$C_{18}H_{16}O_4$	125,092–36-6	–	2933.0	–	–	0.959
37	214.623	6,7-dimethoxy-2-(2-(4-methoxyphenyl)ethyl)chromone▲	$C_{20}H_{20}O_5$	117,596–92-6	–	2978.9	5.188	3.665	2.186
38	219.902	A methoxy and a hydroxy groups as the A-ring and a methoxy group as the B-ring of 2-(2-phenylethyl)chromone▪	–	–	–	–	–	1.733	0.678

Table 11. Identification and relative peak areas of secondary metabolites in agarwood samples induced by complex inducer. ▲ chromone compounds, ○ aromatic compounds, ▪ sesquiterpenes, ☆ others.

amount of alcohol as a solvent, which would cause greater harm to the *A. sinensis*. *A. sinensis* will be affected by the alcohol as a solvent, causing dehydration, drying out, and then death[47]. In subsequent field experiments, when 25% ethanol was used as the negative control solvent, most of the *A. sinensis* trees were damaged, the leaves became less, and the xylem rotted, which also showed the harm of a large amount of alcohol acting on the *A. sinensis*. In the FA group, samples treated with FA (0.05%, 1%) showed higher total peak areas than those treated with 0.5% FA. Similarly, in the A13 group, samples treated with A13 (5%, 15%) had greater total peak areas than those treated with 10% *B. rhodina* A13. Therefore, we used MeJA (0.1%, 1%), FA (0.05%, 1%) and A13 (5%, 15%) for the subsequent experiments.

Design of experiments (DoE) systematically evaluates multiple factors' effects. Both Taguchi's method and factorial design fall under the category of DOE. When dealing with numerous factors, Taguchi's method can be prioritized. It utilizes orthogonal arrays combined with Signal-to-Noise Ratio (S/N Ratio) analysis to identify optimal parameter combinations while mitigating the effects of noise factors. However, Taguchi's method has limitations in interpreting main effects and interaction effects[48]. Factorial design can comprehensively analyze

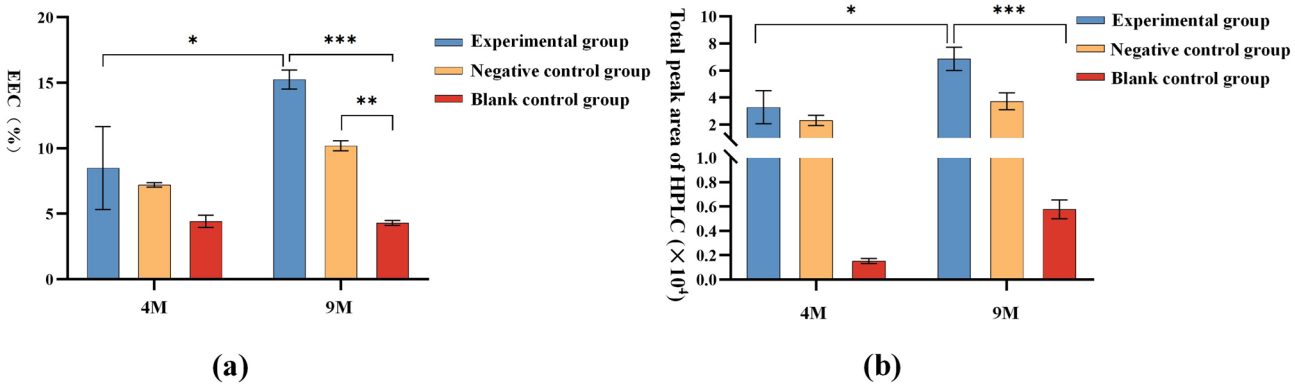

Fig. 7. Bar chart showing the correlation of induction times (**a**) and contents of ethanol-soluble extractives (**b**) with the total HPLC total peak area of characteristic peaks. [1]*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

main effects and interactions at all levels[49]. However, as the number of factors increases, the number of required experiments grows exponentially, leading to computational complexity and difficulties in interpreting the numerous interactions[50]. 2^4 factorial design serves as an efficient and systematic multi-factor optimization tool for situations with limited resources and few factors (≤4), but where a comprehensive analysis of main effects and interactions and efficient screening of key influences are required. This method can simultaneously analyze four factors (A, B, C, D) and all their possible main effects and interactions (such as AB, AC, ABC). Each factor is horizontally and evenly distributed, which can effectively avoid data bias and omission of key influencing factors. Meanwhile, only 16 experiments ($2^4 = 16$) are needed to examine all factor combinations. While quantifying the contribution of each factor to the results through statistical models (such as ANOVA), the synergy or antagonistic effects among the factors can also be revealed, thereby accurately identifying the optimal parameter combinations[51]. This study aims to systematically investigate the contributions of A-MeJA, B-FA, C-A13, and D-Time to agarwood formation and identify the key factors influencing agarwood production. Additionally, GC–MS and HPLC were employed for multi-index validation to compensate for the limitations of a simple two-level analysis. Therefore, this study employed 2^4 factorial design to optimize controllable factors, thereby simplifying the complexity of field experiments while improving agarwood induction efficiency and reducing costs.

The common secondary metabolite in the 16 samples of factorial design experiments was MeJA, in addition to phenolic and fatty acid compounds (Table 5). Their accumulation provides the chemical basis for the special aroma and medicinal value of incense. According to the OPLS-DA analysis results (Fig. 1), the relative contents of MeJA in the chemical compounds of 16 the in vitro branches of *A. sinensis* showed differences at different induction times. It can be seen from Table 4 that the relative contents of MeJA in samples induced for 7 days was higher than that in samples induced for 15 days. In the early stages of induction, the content of MeJA increased rapidly to activate plant defence responses and secondary metabolic pathways. The rapid accumulation of JA in response to wounding has been demonstrated to be essential for the direct defenses at the attack site and during systemic defense signaling[52]. After injury, *A. sinensis* exhibited an early increase in endogenous jasmonic acid compounds, followed by a significant rise in sesquiterpene content[10,53]. It had been further demonstrated that MeJA significantly induced the expression of the sesquiterpene synthase (ASS) gene, which led to an increase in the production of sesquiterpenes[54]. These findings indicated that the JA pathway played a key role in regulating the biosynthesis of sesquiterpene defences in *A. sinensis*. Although only the accumulation and change of JAs were detected due to the short induction time of in vitro branches of *A. sinensis*, the results also suggested that the biosynthesis of sesquiterpene of *A. sinensis* may be promoted at the early stage of inducer treatment. Sesquiterpenes are one of the main chemical components of agarwood, and sesquiterpene content has typically been regarded as an important criterion for the evaluation of agarwood quality[11,55]。

Therefore, in the 2^k factorial design analysis, the relative content of MeJA and the total peak area of each sample were used as indicators to evaluate the effect of inducers. We found that the optimal complex inducer was "1%MeJA + 1%FA + 5%A13 + 7days". However, the effect of A13 at the concentration range of 5%-15% was not significant (Table 6), likely due to that the con-centration was too low. To simplify operating procedures in the field, we used the stock solution of A13 in the field experiment. Moreover, because the in vitro branch of *A. sinensis* was used as a material, the induction time range used in the field experiment was not sufficient. To verify the results of the factorial experiment, we used in vitro branches of *A. sinensis* as experimental materials and evaluated the induction effects of various single inducers and complex inducers. The two algorithms TOPSIS and *OD* value combined the relative content of MeJA and the total peak area of the sample to avoid the bias of single-parameter evaluation (Table 8). The accuracy and reliability of the screening results of 2^k factorial experiments are improved. According to the discriminant analysis of the natural agarwood formation mode, the analysis results show that 57.1% of the natural agarwood formation mode may be integrated (physical injury + chemical damage + fungal induction), and long-term induction factors (fungal induction) are required[26]. Thus, "1%MeJA + 1% FA + A13" was selected as the complex inducer in the field experiments.

Most researchers believe that the longer the duration of artificially induced accumulation, the better the quality of agarwood. However, our previous dynamic study showed that after more than 10 months of artificial

induction, EEC% has begun to have a downward trend[38]. We therefore adopted the 9-month time period and retained part of the *A. sinensis* for follow-up observation. In our experiment, we used agarwood reference drug (standard substances verified by the National Institute for Food and Drug Control to meet the specified requirements) as references. According to the requirements of ChP 2020, we conducted a comprehensive evaluation of wood samples (agarwood) collected from in vivo field experiments. The evaluation includes the description, moisture content, identification, characteristic chromatogram, EEC% and agaroretrol required by ChP 2020. In addition to meeting the requirements of ChP 2020, the similarity of GC–MS and HPLC fingerprints in the experimental group was the highest compared with the agarwood reference drug. Meanwhile, when comparing total peak areas across the three groups, the experimental group also showed the highest total peak area and abundance of compounds. The experimental group's samples are closer to the agarwood reference drug. Therefore, the artificial agarwood induced by complex inducer produced in this study met the requirement of ChP 2020 for medicinal agarwood, and the resin quality reaches the grade IV of agarwood quality[31].

As we know, agarwood primarily contains sesquiterpenes, 2-(2-phenylethyl) chromones, and aromatic compounds. One of the main bases of agarwood's medicinal effects is agarwood essential oil, which chemical composition is mainly sesquiterpenes. Jayachandran et al. analyzed the volatile oil of different grades of *A. malaccensis* and found that the higher the grade of volatile oil, the higher the relative content of sesquiterpenes[56]. Previous studies have shown that natural agarwood is mainly composed of 2-(2-phenylethyl) chromones and sesquiterpenes with their relative content ratio (apparent abundance, *R*) close to 1:1. The ratio of artificial agarwood is between 1.1 and 10.5. The closer the ratio is to 1:1, the more artificial incense can achieve similar levels of chemical composition as natural agarwood[37]. However, the average ratio of blank group samples was 4.28:1, whereas that of the experimental group was 2.28:1. The decrease in ratio may be due to the decrease in 2-(2-phenylethyl) chromones abundance or the increase in sesquiterpenes abundance. However, the EEC% and HPLC results showed that the 2-(2-phenylethyl) chromones content of samples in the experimental group was significantly increased compared to that of samples in the blank group. The content also significantly increased with the increase of induction time (Fig. 7). Therefore, the decrease in the ratio is likely due to an increase in the abundance of sesquiterpenes rather than a reduction of the abundance of 2-(2-phenylethyl) chromones, which suggests that the complex inducer can increase the abundance of sesquiterpenes compounds in agarwood to a certain extent.

We suggest that the complex inducer to produce agarwood not only can satisfy the high demand for natural agarwood, but also can help preserve and protect wild *A. sinensis* tree. At the same time, this provides new ideas for developing efficient artificial agarwood formation technologies and producing high-quality artificial agarwood that closely resembles natural agarwood.

Materials and methods

Materials

Robust and insect-free branches of *A. sinensis* planted in the experimental base of the Tropical Forestry Research Institute, Guangzhou Plantation Base (40° 0′ N, 116° 15′ E) were picked and used in single factor and factorial design experiment for the screening of complex inducer. Resinous parts of wood chips of *A. sinensis* planted in Wugui Mountain (22° 26′ N, 113° 24′ E), Zhongshan, China were collected for in vivo confirmatory experiment. The experimental area belongs to the planting land of Zhongshan Woxianglin Agricultural Development Co., Ltd. The experiments involving the artificial induction of *A. sinensis* and the collection of incense resin were authorized by the company. *A. sinensis* cultivation and agarwood production complied with the Forestry Industry Code of the People's Republic of China (LY/T 3374-2024) issued by the National Forestry and Grassland Administration. All *A. sinensis* trees were identified as *A. sinensis* by Prof. Yan (College of Traditional Medicine, Guangdong Pharmaceutical University, China). *Botryosphaeria rhodina* A13 (EU781670), isolated and provided by associate Prof. Li (Institute of Microbiology, Guangdong Academy of Sciences, China), was used to inoculate on *A. sinensis* trees[57]. Agarwood reference drug (DZYC) was purchased from National Institutes for Food and Drug Control (121222-201203, 121222–202104, Beijing, China). MeJA was purchased from Sigma-Aldrich (#392707, St. Louis, MO). FA, ethanol (EtOH), and trichloromethane (THMS) were purchased from Guangzhou Chemical Reagent Factory (Guangdong, China).

The strain A13 (stored at 4 °C) was cultured on potato glucose agar at 28 °C for 7 days and then inoculated into 500 mL Erlenmeyer flasks, which contained 250 mL potato dextrose broth (potato 20%, glucose 2%, K_2HPO_4 0.3%, $MgSO_4{\cdot}7H_2O$ 0.15%, vitamin B 10 mg/L). After 7 days of cultivation on a 120 r/min rotating shaker at 28 °C, the liquid fermentation broth of the strain A13 was filtered through sterile double-layer gauze fabrics to prepare the stock solution. MeJA, weigh it precisely, add 50% ethanol to dissolve it, and dissolve it to 2% (w/v) at room temperature as inducer I. FA was pipette accurately, and dissolved by adding A13 to give a concentration of 2% (w/v). Finally, A13 was added to 1500 mL and the solution was stirred until evenly mixed to result in inducer II. Then, it was mixed with inducer I, and the final concentration of ethanol, MeJA, FA was adjusted to 25%, 1%, 1% respectively. The final liquid was the complex inducer for in vivo field experiment.

Methods

Establishment of in vitro branch model of A. sinensis

Complex inducers were screened through the use of the in vitro branch model of *A. sinensis* established by a previous study[44]. Under aseptic conditions, fresh branches with a thickness of about 0.5 cm were cut into small sections with a length of about 5 cm. The sections were soaked in 0.1% $HgCl_2$ solution for 5 min and then rinsed 3 times with sterile water. After that, they were soaked in 75% ethanol solution for 5 min and then rinsed with sterilised water to remove residual ethanol. A sterile puncher was used to drill the branches before being placed on a petri dish filled with moist sterile filter paper (Fig. 8).

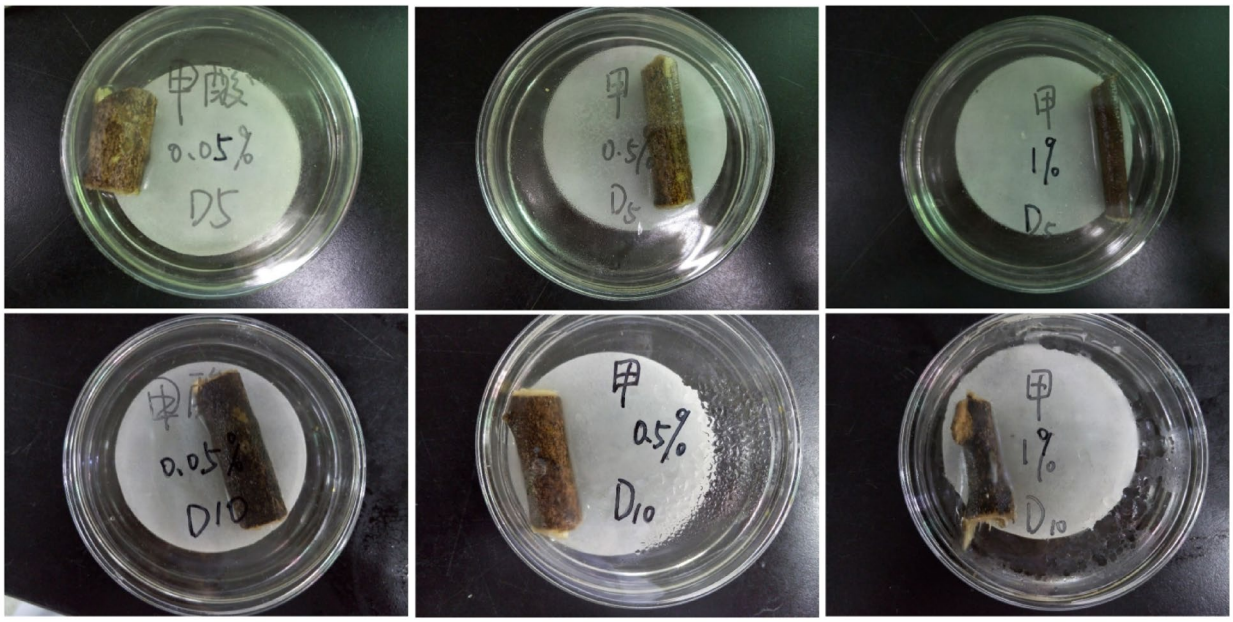

Fig. 8. In vitro induction experiments on excised twig of *A. sinensis* with inducers.

Inducers	Concentration (%)			Time (day)		
	Low	Medium	High			
MeJA	0.1	1	10	5		
FA	0.05	0.5	1	5	7	10
A13	5	10	15	5	7	10

Table 12. Single factor experimental design.

Single factor experimental design
MeJA, FA, and A13 were employed to induce the in vitro branches of *A. sinensis*. Solutions of MeJA (0.1%, 1%, 10% w/v), FA (0.05%, 0.5%, 1% w/v) and A13 (5%, 10%, 15% w/v) were prepared. The solutions were injected into the in vitro branches, which were then sealed with a plastic wrap. The branches were placed in darkness at 28 °C for 5, 7 and 10 days (Table 12).

2^4 factorial experiment design and statistical optimisation
The a^k factorial design was adopted, where *k* is the experimental factor, and a is the factor level, to optimize the design scheme further. The established regression model and multiple regression equation were employed to screen out the best complex inducer more accurately.

According to the results from single factorial analysis and preliminary experiment, MeJA concentration (A; 0.1%, 1%), FA concentration (B; 0.05%, 1%), A13 concentration (C; 5%, 15%) and induction time (D; 7d, 15d) were considered the investigate factors.

Infusion process of complex inducer into A. sinensis
Artificial agarwood from *A. sinensis* was induced by complex inducer through pinhole instillation[17]. A total of 18 batches of *A. sinensis* trees were used in the field experiment and were randomly divided into three groups: experimental group, negative control group, and blank control group. The holes of incense were drilled at 50 and 100 cm from the ground, and the trunk of each *A. sinensis* tree was marked with a label paper (Fig. 9a). After the inducer permeated the *A. sinensis*, they were transported upward through the xylem by transpiration and transported downward through the xylem by gravity. After four and nine months, the trunk was cut down at the hole of incense and then cut horizontally at every 50 cm along the longitudinal axis (Fig. 9b).

HPLC and quality evaluation
High performance liquid chromatography (HPLC) was carried out following the method of Xu et al.[58] and EEC%, agarotetrol content and colour reaction were examined according to the ChP 2020[2] determination of agarwood requirements. GraphPad Prism 8.4.0 and Origin 2021 software were used for significance analysis and to create plots, respectively.

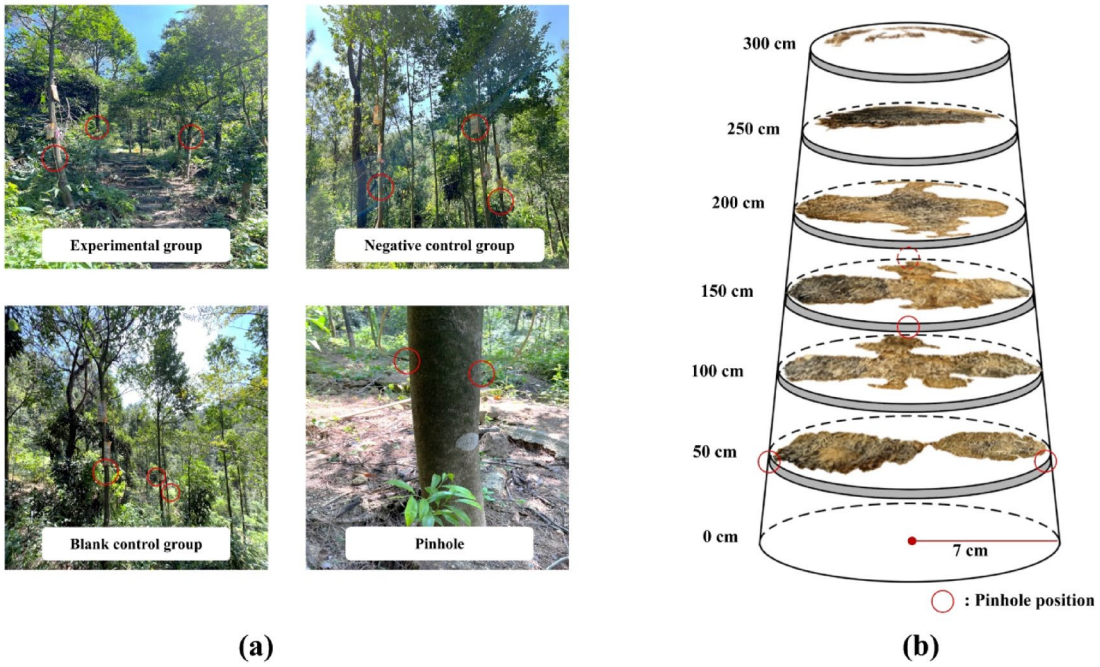

Fig. 9. (**a**) Infusion and (**b**) conduction of fluid in *A. sinensis* tree.

GC–MS fingerprinting and identification of secondary metabolites

GC–MS fingerprinting[37] was employed to determine the content of MeJA in agarwood. Preparation method of test product solution, branches of *A. sinensis* in each test group were placed in a tapered flask with a stopper, in which they were soaked in THMS for 24 h. The extract solution was removed using a rotary evaporator, and the residual was dissolved with THMS before being transferred to a 1 mL volumetric flask. After THMS was dropped to the mark the solution was shaken vigorously and then filtered through a 0.22 μm microporous membrane; and the filtrate was collected. For field verification experiment, all samples were collected and dried at room temperature. Wood samples were ground to powder and then filtered through a 50-mesh sieve. The powder samples of agarwood (0.2 g) were extracted with THMS (10 mL, 24 h) at room temperature. The solvent was evaporated in a water bath (70 °C) until viscous semi-solid masses were obtained. The semi-solid masses were dissolved in the THMS and adjusted to a 2 mL volume. The liquid was shaken vigorously and then filtered through a 0.22 μm microporous membrane. The filtrate was collected and stored in darkness in an air-tight sealed vial at 4 °C. DZYC (0.2 g) was accurately weighed and then subjected to the above steps. The obtained test solutions and standard solution were subjected to subsequent GC–MS analyzes, respectively.

The GC–MS experimental conditions were as follows: GC analysis was performed using the following conditions: For the in vitro branches experiment, GC–MS 7890A-5957C (Agilent) and equipped with a capillary fused silica column HP-5MS (30 m × 0.25 mm × 0.25 μm film thickness, Agilent Technologies); injector temperature: 260 °C; carrier gas (He, 99.999%) flow rate, 1.0 mL min^{-1}; programmed oven temperature, 90 °C for 2 min, then rose at 2.5 °C min^{-1} to 160 °C for 5 min, then rose at 2 °C min^{-1} to 180 °C for 5 min, and last rose at 5 °C ·min^{-1} to 230 °C for 15 min. The split ratio of 1/30; a sample of 1 μL was injected. For field verification experiment, injector temperature: 260 °C; carrier gas (He, 99.999%) flow rate, 1.0 mL min^{-1}; programmed oven temperature, 90 °C for 4 min, then rose at 2 °C min^{-1} to 135 °C for 5 min, then rose at 0.2 °C min^{-1} to 153 °C then 3 °C min^{-1} to 210 °C for 10 min, then rose at 0.5 °C min^{-1} to 230 °C for 20 min, and last rose at 2 °C min^{-1} to 280 °C for 10 min; injections of 1 μL were performed in splitless mode. MS analysis was performed under the following conditions: The samples were operated in the electron ionization (EI) mode (70 eV); ionization voltage, 1 kV; injection port temperature, 250 °C; ion source temperature, 230 °C; solvent delay time, 5 min; quality scan range, *m/z* 50–500.

Mass spectral information of TIC of the samples was obtained using Automated Mass Spectral Deconvolution and Identification System (AMDIS). The information was then compared with the mass spectral information and the retention index (RI) value in the National Institute of Standards and Technology Mass Spectral Library (NIST 17). The relative percentage of agarwood compounds was calculated by the area normalisation method using the GC–MS Postrun Analysis workstation.

Conclusions

In this study, we established the in vitro branch model of *A. sinensis*, which was a simple method compared with the field plant experiments, and was able to screen different inducers at the same time, which greatly shortened the experimental period. Moreover, the in vitro branches of *A. sinensis* were closer to the natural growth condition of the plant. The factorial experimental design is a multi-factor, multi-level, single-effect crossover design, it can accurately evaluate the effect of each independent factor and the interaction between the

factors. The complex inducer was optimized by the factor design experiment, and the best complex inducer was determined to be 1%MeJA + 1%FA + A13. Field experiments proved that the complex inducer could effectively promote the formation of agarwood. This work provides a theoretical basis for optimizing the existing production methods and improving the quality of artificial agarwood. The agarwood formation process is complex, and due to individual differences in each tree, the effectiveness of agarwood-inducing agents can be affected by these factors. In the future, with the application of biotechnology, the optimization of chemical induction technology, and the development of digitalization and intelligence, the problems of unstable quality of agarwood will be solved, and the quality and market recognition of agarwood will be improved, and the standardization and industrialization of agarwood industry will be promoted.

Data availability

The data presented in this study are available on request from the corresponding author.

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Author contributions

Conceptualization, X.G., X.Z., W.Z. (Weiming Zhang) and X.C.; methodology, S.D., B.Q., M.L., X.Z., W.Z. (Weiping Zhou) and X.C.; formal analysis, S.D., Q.D., J.C. and Z.L.; resources, W.Z. (Weiming Zhang); writing—original draft preparation, S.D.; writing—review and editing, X.G. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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