*Pak. J. Bot.*, 51(5): 1645-1651, 2019. DOI: http://dx.doi.org/10.30848/PJB2019-5(16)

# EXPERIMENTAL INDUCTION OF 2-(2-PHENYLETHYL) CHROMONE IN AERIAL ROOTS OF *AQUILARIA SINENSIS*

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**Abstract**

Agarwood, the valuable spice, is the resin-containing wood formed in *Aquilaria, Gonystylus* and *Gyrinops* spp. However, it is facing the problem of wild resource depletion and insufficient artificial production. Investigation and elucidation of the induction and formation processes of the marker ingredients of agarwood, such as sesquiterpenes and chromones, is key to solving this problem. Unlike other studies that have paid more attention to the formation of sesquiterpenes in fresh wood or suspension systems, the present study focused on constructing an aeroponic cultivation system and using it for chromone induction. The experimental results showed that optimal rooting rates and amounts were obtained by cutting off the lateral roots of soil-grtown *Aquilaria sinensis* seedlings, treating them with 1.5g·L$^{-1}$ IBA and placing them in the water aeroponic cultivation system. The lateral roots cultivated in 1.2 dS·m$^{-1}$ MS medium grew vigorously and accumulated the maximum biomass. The GC/MS results showed the formation and continued enrichment of 2-(2-phenylethyl) chromone starting on the fifth day after treatment with methyl jasmonate (MeJA) (50 μM). Therefore, the present study primarily established an aeroponic cultivation system for aerial rooting of *A. sinensis* that can be used for studying chromone induction; this study also extended the experimental ability for determining how the other marker ingredients of agarwood, chromones, are generated.

**Keywords:** *Aquilaria sinensis*, Lateral root, 2-(2-phenylethyl) chromone, Aeroponic system

**Abbreviations:** MeJA---methyl jasmonate, NAA--- naphthalene acetic acid, IBA--- indole-3-butytric acid, ABT[1]---20% naphthaleneacetic acid & 30% indole-3-acetic acid

## Introduction

Agarwood is a black-resin wood formed from the stems, branches or roots of trees belonging to the species *Aquilaria*, *Gonystylus* and *Gyrinops* after damage (due to natural causes such as wind, lightning, insect bites or microbial infection), and are considered to be a pathological product (Okudera & Ito, 2009; Xu *et al.*, 2013). As a precious spice, agarwood is not only widely respected by global religions such as Buddhism and Christianity but also valuable for health care and collection. As a traditional medicine, this resin-containing wood can be used to make antiemetics, sedatives and digestive drugs (Naef, 2011; Mei *et al*., 2013; Xu *et al*., 2013; Espinoza *et al.*, 2014). However, the current endangered status of wild agarwood resources and the low efficiency of artificial production makes it difficult to meet market demand (Zhang *et al*., 2010; Espinoza *et al*., 2014; Siah *et al*., 2016).

For many years, numerous researchers committed themselves to the development of efficient production technology for agarwood; some progress has been made based on the exploration and clarification of the synthetic process of the marker ingredients of agarwood, sesquiterpenes and chromones. Ito (2005) and Liu (2015) showed that both methyl jasmonate (MeJA) and $H_2O_2$ could induce the production of the sesquiterpenes α-guaiene, δ-guaiene and α-humulene in suspension cells of *Aquilaria sinensis*. Using molecular biology techniques, Xu (2013) showed that sesquiterpene synthase (*ASS*$_{1\text{-}3}$) was the main synthase involved in the synthesis of δ-guaiene. Compared with the reported studies on sesquiterpenes, the study of chromones has been limited (Affenzeller *et al*., 2009), which has hampered understanding of the entire process of agarwood production. In addition, if fresh wood is used as the study material, the experimental cycle is long, and the environmental factors are complex and uncontrollable (Affenzeller *et al*., 2009; Mei *et al*., 2013; Yang *et al*., 2016). Currently, some reasearchers began to pay attention on aeroponic materials (Vaughan *et al*., 2011; Kumari *et al*., 2016).

The use of suspended cells as the material is not very compatible with simulation of the natural process of agarwood production due to their essential characteristics of heterotrophy and being an *in vitro* system. The present study attempted to make use of an aeroponic cultivation system with a short cycle and controllable conditions for studying the induction of chromone formation in *A. sinensis*.

## Materials and Methods

**Materials and aeroponic cultivation system:** The starting material of this study was one-year-old *A. sinensis* seedlings which were approximately 40 cm in height and 0.5 cm in diameter. The aeroponic cultivation system was established in our laboratory (Fig. 1A, B). The aeroponic chamber was 52 cm × 42 cm × 22 cm (length × width × height) with black and white shading film. The transparent cover was 50 cm × 40 cm × 30 cm. The system was provided with 3 L of pure water or Murashige & Skoog (MS, 1962) medium circulated by the PE pipe with an inner diameter of 8 mm. The spray cycle was set at 30 min off / 5 min on. The room temperature was controlled at 25-27°C, and the humidity was above 60 %.


Fig. 1. Aeroponic unit. A, *A. sinensis* seedlings were planted in the aeroponic chamber with a transparent plastic cover (Scale bar, 10 cm); B, Profusion of adventitious roots within the aeroponic chamber (Scale bar, 3 cm); C~E, Lateral roots under different EC (0.6, 1.2, 2.4 dS $m^{-1}$) cultivation (Scale bar, 2 cm).

**Induction and cultivation of aerial rooting:** The seedlings were removed from the nutrition bag. After cleaning the soil from the roots, all of the lateral roots were cut off, followed by disinfection of the main root with 0.1% $KMnO_4$ for 1 min. The 10-s treatments with different growth regulators (NAA, IBA and ABT[1]) at different concentrations (0.5, 1.0 and 1.5 g·$L^{-1}$) were designed for the induction of the aerial rooting. After the roots were dipped in the solution, the seedlings were immediately transferred into the aeroponic cultivation system, and the related indicators were recorded on the 20th day of cultivation in pure water. After rooting had occurred, the water was replaced with MS media of different electrical conductivities (0.6, 1.2 and 2.4 dS·$m^{-1}$) to compare their effects on rooting, and the related indicators were recorded and analyzed on the 25th day.

**Stress treatment and sample preparation of the aerial rooting:** After 25 days of lateral rooting, 50 µM MeJA solution was added to the original cultivation medium as a stress treatment to induce the production of chromone metabolites. After zero, 3, 5 and 7 days of treatment, the lateral roots were collected for testing, and the *A. sinensis* standard medicines (SM, purchased from the National Institutes for Food and Drug Control, Beijing, China) served as the positive control. For sample processing, 0.5 g of sample was weighed precisely (dry weight) and immersed in 20 mL ether (for 24 h), and the procedure was repeated once more. The 40 mL of combined extracts were filtered, and the solvent was evaporated under reduced pressure to obtain an oily brown precipitate. The precipitate was dissolved in ether to a volume of 1 mL for GC-MS.

**GC-MS analysis of chromone derivatives in aeroponic roots of *A. sinensis*:** For gas chromatography, a quartz capillary column (HP-5ms; Agilent Technologies, Santa Clara, CA, USA) with dimensions of 30 m × 0.25 mm and a film thickness of 0.25 µm was used. The following temperature program was used: the initial temperature of 90°C was increased to 220°C at a rate of 5°C·$min^{-1}$ and then to 280°C at a rate of 3°C $min^{-1}$; the final temperature was maintained for 15 min. The injection volume was 5 µL in a splitless port, and the volatilization chamber temperature was 250°C; the carrier gas was He of high purity (99.99%). The following conditions for MS were used: electron impact ion source; quadrupole temperature of 150°C; electron multiplier voltage of 2165 V; interface temperature of 280°C; solvent retention time of 3 min; and mass fragments were scanned in the range of 50-550 (m/z).

After hormone treatment and cultivation in pure water for 20 days, the rooting rates and amounts were measured; three replicates were performed for each group with 15 seedlings per replicate. The total root length and fresh weight of each plant were measured after cultivation in the nutrient medium for 25 days following the initiation of the lateral rooting (five seedlings per group). The ether extracts were subjected to GC-MS (with three replicates for each group and a mixed lateral rooting sample of six seedlings per replicate).

**Statistical analyses:** All data were analyzed using SPSS v.16 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to compare the means by Tukey's test (Haynes, 2013). All results are presented as the mean value ± standard deviation (SD).

## Results

**Effect of hormone treatment on lateral rooting:** Lateral rooting has become a good system for investigating the growth and secondary metabolism of some important economic crops, and its induction is a complex process. In this study, the results of the treatments of IBA and ABT[1] were good in terms of their effect on the rooting rate of *A. sinensis* (Table 1). The rooting rate of *A. sinensis* after treatment with 0.5 g·$L^{-1}$ IBA was the highest (86.67%), but there was no significant difference in general. For the highest

rooting amount, the optimal condition was 1.5 $g \cdot L^{-1}$ IBA, which produced a rooting amount of 32.87 $root \cdot plant^{-1}$; this rooting amount was higher than rooting amounts obtained with other IBA concentrations or other hormones. NAA was not suitable for the rooting induction of *A. sinensis*, and the two indexes of the treatment group were only slightly higher than the indexes of the control group. In addition, the lateral root bulge was also observed seven to eight days after IBA treatment, which is nearly twice as early as the other treatment groups. The results of this study also demonstrated that IBA is suitable for the rooting induction of *A. sinensis* at an optimal IBA concentration of 1.5 $g \cdot L^{-1}$.

**Effect of electrical conductivity on lateral rooting:** This study showed that low electrical conductivity (EC) could facilitate plant growth and development. For example, tomato and peppermint displayed maximum fresh weights in 1.4 $dS \cdot m^{-1}$ medium. However, high EC (2.4 $dS \cdot m^{-1}$) inhibited the accumulation of root biomass. The results showed that the rooting of *A. sinensis* was significantly different at different EC levels (0.6, 1.2 and 2.4 $dS \cdot m^{-1}$) (Table 2), and the maximum fresh weight (1272.6 ± 681.6 $mg \cdot plant^{-1}$) and longest root length (89.7 ± 25.7 $cm \cdot plant^{-1}$) were obtained at 1.2 $dS \cdot m^{-1}$ (Fig. 1C, D), which ccould be used as the reference value for favoring the rooting of *A. sinensis*. Further reduction of EC (0.6 $dS \cdot m^{-1}$) was not conducive to biomass accumulation of the root. The high limit of EC can be set at 2.4 $dS \cdot m^{-1}$ (Fig. 1E) because at this level, the indices of root length and fresh weight were significantly decreased compared with those indexes at an EC of 1.2 $dS \cdot m^{-1}$.

**Formation of chromone derivatives in the aerial rooting of *A. sinensis*:** GC-MS was used to detect lateral rooting at different time points after induction with 50 μM MeJA. The results showed that in the untreated samples (zero days) and samples treated for three days, chromone as a marker ingredient of *A. sinensis* agarwood was not detected. After five days of treatment, 2-(2-phenylethyl) chromone was detected in the lateral root sample, while the relative content was increased in the sample that had been treated for 7 days, indicating it's a continual accumulation of chromones (Fig. 2 & Table 3). The results showed that not only 2-(2-phenylethyl) chromone but also a variety of hydrocarbons and aromatic substances were induced in aerial roots under MeJA constantly stress.

**Table 1. Effect of auxin on rooting of *A. sinensis* (N=15).**

| Auxin concentration ($g \cdot L^{-1}$) | % of rooted seedlings (mean ± SE) | Number of roots per seedling (mean ± SE) |
|---|---|---|
| CK (pure water) | 0e | 0a |
| NAA ($g \cdot L^{-1}$) | | |
| 0.5 | 26.67 ± 6.67d | 2.47 ± 2.73a |
| 1.0 | 6.67 ± 0.00e | 0.40 ± 0.46a |
| 1.5 | 2.22 ± 3.85e | 0.13 ± 0.23a |
| IBA ($g \cdot L^{-1}$) | | |
| 0.5 | 86.67 ± 6.67a | 9.93 ± 5.76a |
| 1.0 | 84.44 ± 10.20ab | 6.38 ± 3.11a |
| 1.5 | 84.44 ± 10.18ab | 32.87 ± 15.14a |
| ABT[1] ($g \cdot L^{-1}$) | | |
| 0.5 | 84.44 ± 10.20ab | 13.07 ± 8.02a |
| 1.0 | 73.30 ± 20.00abc | 10.93 ± 5.85a |
| 1.5 | 75.57 ± 10.19abc | 13.17 ± 5.52a |

Data were recorded 20 days after exogenous hormone treatments and planting. Each mean is based on three replicates, each of which consisted of 15 seedlings. Values are the means of three independent experiments. Mean values marked by different letters for different treatments within a column are significantly different from one another at $p<0.05$

**Table 2. The effect of EC levels on the root characteristics of *A. sinensis* (N=5).**

| EC ($dS\ m^{-1}$) | FW ($mg\ plant^{-1}$) | Total root length ($cm\ plant^{-1}$) | Relative water content (%) |
|---|---|---|---|
| 0.6 | 473.7 ± 324.5ab | 39.2 ± 25.2b | 86.92 ± 1.22a |
| 1.2 | 1272.6 ± 681.6a | 89.7 ± 25.7a | 88.76 ± 1.41a |
| 2.4 | 91 ± 95.1b | 6.4 ± 6.5b | 87.93 ± 2.18a |

Data were recorded after 25 days of cultivation. Mean values marked by different letters for different treatments within a column are significantly different from one another at $p<0.05$

**Table 3. Main chemical constituents of ether extracts from experiment samples and standard medicines (N=3).**

| # | Compound | Formula | Molecular mass | Relative content (%) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Experiment samples | | | | Standard medicnes |
| | | | | 0d | 3d | 5d | 7d | |
| 1. | Dibutyl phthalate + | $C_{16}H_{22}O_4$ | 278 | - | 5.63 ± 2.06a | 4.76 ± 0.59a | 2.45 ± 0.41b | - |
| 2. | 3-Pentanone, 1,5-diphenyl- + | $C_{17}H_{18}O$ | 238 | - | - | - | 0.46 ± 0.12a | - |
| 3. | 1-Penten-3-one,1,5-diphenyl-+ | $C_{17}H_{16}O$ | 236 | - | - | - | 0.21 ± 0.06a | - |
| 4. | Hexadecane# | $C_{16}H_{34}$ | 226 | - | 2.06 ± 0.33a | 2.28 ± 1.82a | 0.21 ± 0.03b | - |
| 5. | 1-Dodecanol | $C_{12}H_{26}O$ | 186 | - | 1.36 ± 0.75a | - | - | - |
| 6. | Eicosane# | $C_{20}H_{42}$ | 282 | - | - | 2.00 ± 0.16a | 9.68 ± 2.71b | - |
| 7. | 2-Phenethyl-4H-chromen-4-on* | $C_{17}H_{14}O_2$ | 250 | - | - | 2.10 ± 1.18a | 2.63 ± 0.11a | 0.32 |
| 8. | Pentadecanal- | $C_{15}H_{30}O$ | 226 | - | 0.27 ± 0.06a | 0.73 ± 0.18b | 0.34 ± 0.02a | - |
| 9. | 1-Hexadecanol | $C_{16}H_{34}O$ | 242 | - | 1.42 ± 0.77a | 1.42 ± 0.77a | 0.42 ± 0.05b | - |
| 10. | Heneicosane# | $C_{21}H_{44}$ | 296 | - | - | 1.43 ± 0.99a | 1.43 ± 0.42a | - |
| 11. | Phthalic acid,di(2-propylpentyl) ester | $C_{24}H_{38}O_4$ | 390 | - | - | - | 1.16 ± 0.02a | - |
| 12. | Tetracosane | $C_{24}H_{50}$ | 338 | - | - | 2.66 ± 1.23a | 3.28 ± 0.48a | - |
| 13. | 4H-1-Benzopyran-4-one,5-hydroxy-7-methoxy-2-(4-methoxyphenyl)- | $C_{17}H_{14}O_5$ | 298 | 0.71 ± 0.21a | 1.59 ± 0.43b | 0.64 ± 0.11a | 0.39 ± 0.18a | - |
| 14. | 2-Butanone,4-phenyl- + | $C_{10}H_{12}O$ | 148 | 1.75 ± 0.61a | - | - | 1.59 ± 0.25a | 15.57 |
| 15. | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256 | 3.52 ± 1.47a | - | - | - | - |
| 16. | Docosanal | $C_{22}H_{44}O$ | 324 | 0.79 ± 0.07a | - | - | - | - |
| 17. | Stigmasterol | $C_{29}H_{48}O$ | 412 | 16.67 ± 0.91a | - | - | - | - |
| 18. | 4',5-Dihydroxy-7-methoxyflavanone | $C_{16}H_{14}O_5$ | 286 | - | 0.54 ± 0.13a | 0.52 ± 0.11a | 1.07 ± 0.54a | - |
| 19. | Bis(2-ethylhexyl) phthalate + | $C_{24}H_{38}O_4$ | 390 | - | 1.99 ± 0.75a | - | - | - |
| 20. | 1,14-Tetradecanediol | $C_{14}H_{30}O_2$ | 230 | - | 0.33 ± 0.09a | - | - | - |
| 21. | 6,7-Dimethoxy-2-phenethyl-4H-chromen-4-one* | $C_{19}H_{18}O_4$ | 310 | - | - | - | - | 7.23 |
| 22. | 6,7-Dimethoxy-2-(4-methoxyphenethyl)-4H-chromen-4-one* | $C_{20}H_{20}O_5$ | 340 | - | - | - | - | 0.37 |
| 23. | 6-Methoxy-2-phenethyl-4H-chromen-4-one* | $C_{18}H_{16}O_3$ | 280 | - | - | - | - | 0.53 |

Note: * indicates 2-(2-phenylethyl) chromone, + indicates aromatic compounds, # indicates alkane compounds, - indicates not detected. Mean values marked by different letters for different treatments within a column are significantly different at $p<0.05$

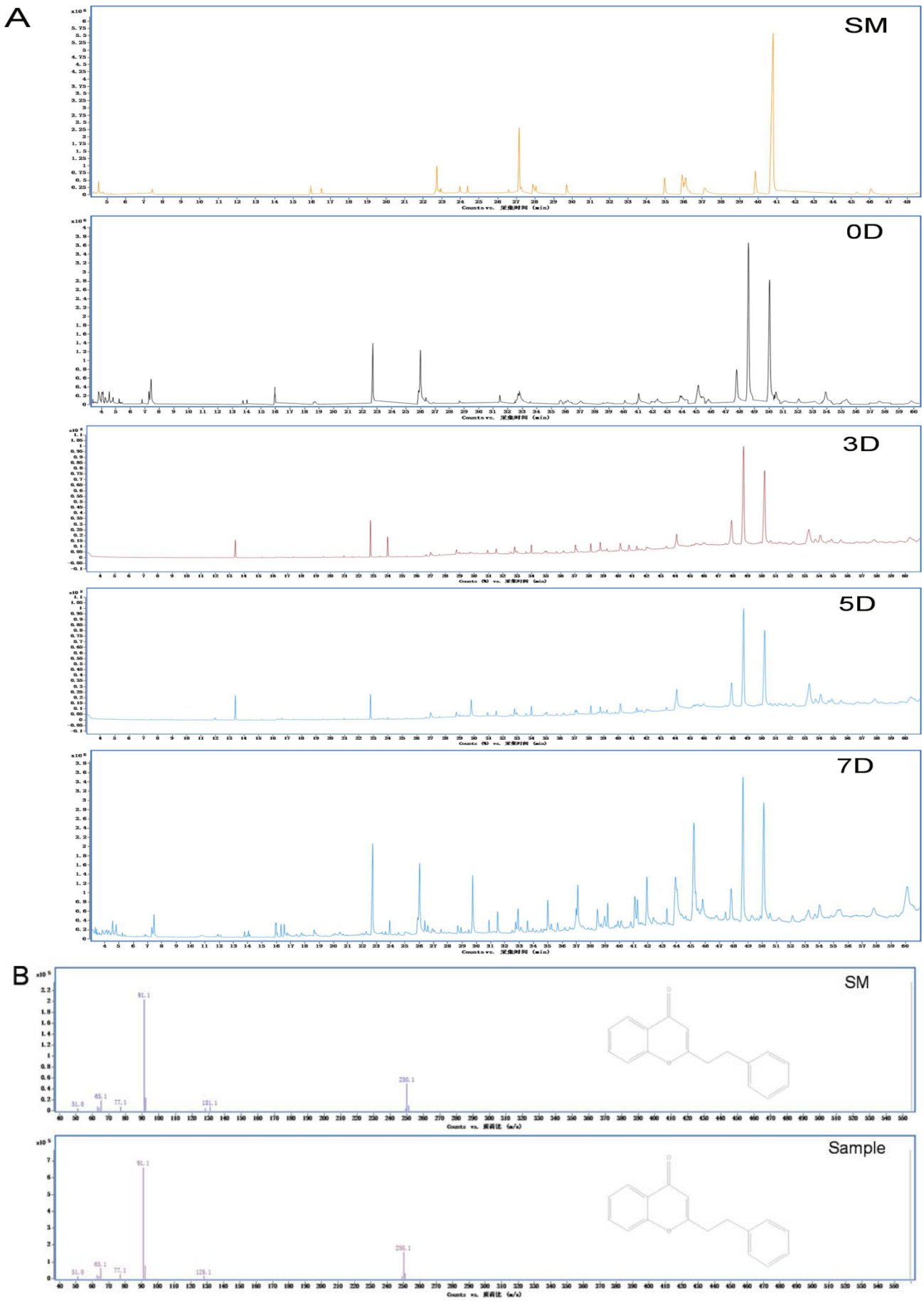


Fig. 2. GC-MS profiles. A, Total ion chromatogram of the different samples; B, Mass spectra of the 2-(2-phenylethyl) chromone from standard medicines(SM) and experiment samples.

## Discussion

In general, the regeneration of adventitious roots of plants must be stimulated by hormones, and plant growth regulators can regulate endogenous hormones related to adventitious root formation, especially woody plants such as *A. sinensis*. As the main factor in this process (Ahkami *et al*., 2013), exogenous hormones can improve both the rooting rate and amount (Kesari *et al*., 2009). However, there are differences in the formation of adventitious roots by different kinds of regulators. As reported in the literature, IBA is a highly effective, non-toxic and stable artificial hormone that can induce the rooting of most species (De Klerk *et al*., 1997; Ludwig-Müller, 2003; Henrique *et al*., 2006), and the amount of rooting is also increasing with the increase of its concentration, but for the best result of 1.5 $g \cdot L^{-1}$, which needs to further investigate.

NAA is also a widely used regulator. The rooting rate and amount are not ideal when the concentration is increased in the experiment, indicating that it is not suitable for the production of adventitious roots of *A. sinensis*. Besides, ABT[1] includes 20% naphthaleneacetic acid and 30% indole-3-acetic acid (IAA), we speculate this result should be promoted by IAA, which has a central function in the formation and development of adventitious roots (Liu & Reid, 1999)**.** The hormone IBA is also an endogenous auxin that has been transformed from IAA (Van der Krieken *et al*., 1993) that is the reason why ABT[1] group is more effective than NAA group. It is worth noting that seedlings with the same initial appearance showed large differences in lateral rooting, which was not conducive to uniform sampling in subsequent experiments. This problem needs to be further investigated.

Electrical conductivity is an important factor affecting the biomass accumulation of lateral roots in an aeroponic cultivation system (Montesano *et al*., 2010). The suitable EC is very important for the growth and development of adventitious roots, too low EC leads to malnutrition and high EC (5 $dS \cdot m^{-1}$) leads to high intracellular osmotic pressure and dehydration in adventitious roots, thereby inhibiting their growth (Tabatabaie & Nazari, 2007; Cui *et al*., 2010; Montesano *et al*., 2010; Lee & Paek, 2012). It is worth noting that seedlings with the same initial appearance showed large differences in lateral rooting, not conducive to uniform sampling in subsequent experiments. This problem needs to be further investigated.

Research reports pointed out that 2-(2-phenylethyl) chromone only can be produced after stress injuries (Wang *et al*., 2016). This study showed the production of 2-(2-phenylethyl) chromone in *A. sinensis* aeroponic roots during MeJA stress 5 and 7 days. During the experiment, it was found that the process of lateral root formation and the accumulation of chromone is coincident with the process of lysigenous aerenchyma formation in the lateral roots (unpublished data), speculating that the formation of 2-(2-phenylethyl) chromone could be associated with the death toll of cells (Drew *et al*., 2000; Mei *et al*., 2013). Hydrocarbons and dibutyl phthalate (Table 3) have certain toxic effects (Cui *et al*., 2013), so we think that could be the cause of programmed cell death and related to chromone production at the same time.

In summary, we think that exogenous MeJA and possible endogenous jasmonic acid may be the main factors of induction (Xu *et al*., 2013; Xu *et al*., 2016), although our results did not induce more kinds of chromones as standard medicines (Table 3), we believe that stimulation by multiple induction factors could be achieved. We have preliminarily established an aeroponic cultivation system for *A. sinensis*, and exogenous MeJA was used to successfully induce the formation of chromones, which provided an effective new method for the further study of secondary metabolites of the marker ingredients of *A. sinensis* agarwood with molecular biology.

## Acknowledgements

We are grateful to the research groups of Prof. Li Lin at Guangzhou University of Chinese Medicine for the assistance in GC-MS methods. The study was supported by the grants of National Natural Science Foundation of China (No.81102764), Special fund project of college discipline and specialty construction in Guangdong province of China (2013CXZDA011), and Regular Institution of Higher Education Innovative Team Project of Guangdong Province - Innovative research team of traditional Chinese medicine resources (2016KYTD02).

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(Received for publication 4 May 2018)