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Establishment of Hairy Root Culture System in Atractylodes chinensis for Enhanced Production of Medicinal Sesquiterpenoids

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Abstract

Atractylodes chinensis (DC.) Koidz., a pharmacologically significant medicinal plant with various metabolites, produces sesquiterpenoids like atractylodin and *β*-eudesmol that dictate its medicinal quality and underpin its clinical efficacy. While hairy root systems offer industrial potential for metabolite production, no such system existed for *A. chinensis*. Here, we report the successful establishment of an efficient hairy root induction system for *A. chinensis* using *Agrobacterium rhizogenes* ATCC15834 with leaf and petiole explants, achieving higher induction rates than strains R1000 30 and LBA9402. Optimal growth occurred on 1/2 MS solid medium supplemented with 0.5 mg·L-1 IAA, 0.1 mg·L-1 KT, and 5% sucrose, yielding maximal biomass and metabolite accumulation over a 52-day culture period. Among the established hairy root lines, those induced from petioles (particularly line P-6) exhibited superior biomass and significantly higher levels of key metabolites, including atractylodin, *β*-eudesmol, total polyphenolic acids, and total flavonoids at day 52, compared to the leaf-induced line (L-1). Transcriptomic analysis confirmed the key genes involved in *β*-eudesmol and atractylodin biosynthesis upregulated relative to wild plants. Furthermore, treatment of the hairy roots with melatonin (MT) or its chemical homologs (5- methoxyindole, 5-MI; 5-methoxytryptamine, 5-MT) demonstrated that only 0.5 mmol·L^{-1} 5-MI significantly enhanced *β*-eudesmol production. Remarkably, the *β*-eudesmol content achieved at 168 hours post-treatment was 1.82-fold higher than that in wild 3-year-old *A. chinensis* roots. This study establishes a robust platform for *A. chinensis* hairy root culture and highlights 5-MI as a potent inducer, providing a valuable foundation for the industrial scale production of its medicinally active compounds.

1. Introduction

Atractylodes chinensis (DC.) Koidz, a perennial herb of the Asteraceae family, is a cornerstone species in Traditional Chinese Medicine (TCM), traditionally termed "Cangzhu". Native to northern China (Inner Mongolia, Shaanxi, Jilin), it is revered for its therapeutic properties in dispelling dampness, fortifying the spleen, and alleviating rheumatic disorders (Cho et al., 2016, Gao et al., 2023, Wang et al., 2023). Modern pharmacological researches have shown that the sesquiterpenoids compounds including atractylodin and *β*-eudesmol, are the main ingredients of its pharmacological activities in *A. chinensis* in clinical practice, which possess anti-inflammatory, antibacterial, antiviral, hepatoprotective and anti-tumor effects (Kimura & Sumiyoshi, 2012, Cheng et al., 2016, Kim et al., 2016a, Xie et al., 2018). Atractylodin has a wide range of pharmacological effects and great potential for development and application. In recent years, it had been verified that atractylodin activated DRD2 to attenuate motor deficits and gait disturbances, which protected dopaminergic neurons in Parkinson's disease mice (Li et al., 2022), demonstrating the potential of atractylodin as a neuroprotective agent (Wang et al., 2023). As well as *β*-eudesmol, it had been shown to inhibit the growth of cancer cells (Ma et al., 2008) and had a regulatory effect on the gastrointestinal tract (Kimura & Sumiyoshi, 2012). Besides, *β*-eudesmol was found to significantly inhibit mast cell migration and reduce F-actin formation in a concentration-dependent manner (Nam et al., 2017).

Due to its medicinal value, the market demand for *A. chinensis* is rapidly increasing, and the wild resource are insufficient, which many regions begin to cultivated *A. chinensis*. However, the accumulation of active metabolites such as atractylodin, *β*-eudesmol, atractylone, volatile oils, atractylenolide I, atractylenolide II and atractylenolide III in *A. chinensis* cultivated plants require 3–5 years to accumulate peak metabolite levels (Liu et al., 2024). So, developing standardized production of metabolites system is important. Root is commonly used tissue of Traditional Chinese Medicine, and also the main location to product secondary metabolites. Hairy roots are induced roots that synthesized various secondary metabolites, which are induced by *Agrobacterium rhizogenes* (Chandra & Chandra, 2011). Hairy roots have the effects of fast growth rate, stable genetic characteristics, and high production of secondary metabolites (Guillon et al., 2006). Hairy root cultures, induced by *Agrobacterium rhizogenes*, offer a scalable solution for metabolite biosynthesis. These systems combine rapid growth, genetic stability, and high secondary metabolite yields (Guillon et al., 2006), with established success in over 100 medicinal plants, including *Panax ginseng*, *Brassica napus*, *Saliva miltiorrhiza*, *Astragalus membranaceus*, *Raphanus sativus*, etc. (Banerjee et al., 2012, Bai et al., 2020, Balasubramanian et al., 2018, Jiao et al., 2015). However, within the Asteraceae family, only *A. lancea* has reported hairy root induction (Zhang et al., 2023a). No protocols exist for *A. chinensis*, hindering industrial exploitation.

Various chemical inducers are employed to enhance the production of plant secondary metabolites. Among these, melatonin (MT, N-acetyl-5-methoxytryptamine), a biologically active small molecule ubiquitous in plants, functions as an effective inducer that promotes the accumulation of secondary metabolites (Kim et al., 2016b). Jahan et al. (Jahan et al., 2020) found that exogenous MT treatment significantly increased the content of phenols, flavonoids, and anthocyanins in tomato seedlings. Another study had shown that the addition of exogenous MT significantly increased the total glucosinolate content of broccoli (Wei et al., 2020). Spraying MT resulted in the highest total isoflavone content in soybeans on the 5th day of germination (Wu et al., 2023). Despite its widespread presence in plants, MT's low endogenous concentrations and complex biosynthetic pathway significantly limit its practical application as an inducer in agriculture. Consequently, identifying low-cost, effective, and highly efficient functional substitutes for melatonin holds considerable value for future agricultural practices. One promising candidate is 5-methoxyindole (5-MI), a chemical homolog and synthetic intermediate of MT. 5-MI offers advantages such as low cost and high synthetic efficiency, suggesting good potential for development and application (Kong et al., 2021). However, the molecular mechanism underlying the induction of secondary metabolites and bioactive compounds in *A. chinensis* by MT and its chemical homologs like 5-MI remain unclear.

Herein, we establish the hairy roots induction system for *A. chinensis* using *A. rhizogenes* ATCC15834. In addition, we optimized medium to grow *A. chinensis* hairy roots. We systematically evaluated petiole- and leaf-derived explants, established growth kinetics, and quantified sesquiterpenoid productions, such as atractylodin, *β*-eudesmol, total flavonoid, total polyphenolic acid, and transcriptional profiling of biosynthetic genes. By benchmarking metabolite yields against wild 3-year-old plants, this work bridges critical gaps in *A. chinensis* biotechnology, enabling sustainable production of its pharmacologically vital compounds. Additionally, melatonin (MT) and two chemical homologs of MT, 5-Methoxyindole (5-MI)

and 5-Methoxytryptamine (5-MT) were used to treat *A. chinensis* hairy roots, and detected the expression levels of genes related to secondary metabolic pathways and the content of secondary metabolites. We explore the changes in the composition of metabolites in *A. chinensis* induced by MT and chemical homologs, which enrich the understanding of the important role of MT induced plant production of secondary metabolites.

2. Materials and Methods

2.1 Plant Materials

The sterile seedlings of *Atractylodes chinensis* (DC.) Koidz were saved in our laboratory. Seeds and wild *A. chinensis* were collected in Chifeng City, Inner Mongolia, China (geographic coordinates: 41°17'10"N – 45°24'15"N, 116°21'07"E – 120°58'52"E) in September 2022.

2.2 Establishment of hairy roots transformation

Petiole and leaf explants of *A. chinensis* were prepared: leaves were sectioned into 1 cm × 1 cm fragments, and petioles were trimmed to 1 cm lengths. Explants were pre-cultured on MS solid medium (pH 5.8) in darkness at 25°C for 12 h. *Agrobacterium rhizogenes* strains ATCC15834, R1000 and LBA9402 were cultured in YEB medium until reaching OD_{600} = 0.6 at 28°C. Pre-treated explants were transferred in *A. tumefaciens* containing 300 μM Acetosyringone and co-cultivated at 28°C (150 rpm) for 30 min. After co-cultivation, tissues were transferred to MS solid medium supplemented with 500 $mg{\cdot}L^{-1}$ cefotaxime (cef) and incubated in darkness at 25°C for 7 d. The tissues were washed with sterile water and replaced onto fresh MS solid medium every week, which the concentration of cef was gradually reduced to 0 $mg{\cdot}L^{-1}$.

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Total genomic DNA of *A. chinensis* hairy roots were extracted using a Plant Genomic DNA Kit (Tiangen, Beijing, WI, China). Specific primers of *rolB* and *rolC* were designed to detected the Ri plasmid of *A. tumefaciens* (Shkryl et al., 2023, Djerdjouri et al., 2024) (Table S1). After PCR amplification, the PCR reaction mixtures were loaded directly onto 1% agarose gel for electrophoretic analysis.

2.3 Optimization of medium of hairy roots

Once the hairy roots grew out, the roots were transferred on 1/2 MS solid medium and kept in the darkness at 25°C. However, the growth was slow. To increase the growth rate of *A. chinensis* hairy roots, different combination of hormones was added to 1/2 MS or 6,7-V solid medium, which were 1/2 MS + 0.5 $mg{\cdot}L^{-1}$ IAA + 0.1 $mg{\cdot}L^{-1}$ KT, 1/2 MS + 0.5 $mg{\cdot}L^{-1}$ NAA + 0.1 $mg{\cdot}L^{-1}$ KT, 1/2 MS + 0.5 $mg{\cdot}L^{-1}$ 6-BA + 0.1 $mg{\cdot}L^{-1}$ KT, 6,7-V + 0.5 $mg{\cdot}L^{-1}$ IAA + 0.1 $mg{\cdot}L^{-1}$ KT, 6,7-V + 0.5 $mg{\cdot}L^{-1}$ NAA + 0.1 $mg{\cdot}L^{-1}$ KT and 6,7-V + 0.5 $mg{\cdot}L^{-1}$ 6-BA + 0.1 $mg{\cdot}L^{-1}$ KT. Each medium was supplemented with 3% or 5% sucrose. About 0.08 g FW of hairy roots were transferred to 90 mL liquid medium in 150 mL erlenmeyer flasks, and

cultured for 60 d prior to biomass assessment to determine the optimal medium for growing *A. chinensis* hairy roots.

About 0.3 g FW of hairy roots were cultured in 90 mL 1/2 MS + 0.5 mg·L^{-1} IAA + 0.1 mg·L^{-1} KT + 5% sucrose medium in 150 mL erlenmeyer flasks. Fresh weight (FW) was recorded at 0, 8, 16, 24, 32, 36, 40, 44, 48 and 52 d to establish growth kinetics.

2.4 Determination of metabolites of *A. chinensis* hairy roots

The *A. chinensis* hairy roots were selected at 0, 8, 16, 24, 32, 36, 40, 44, 48 and 52 d. Hairy roots were washed and dried at 37°C. For determinating of atractylodin and *β*-eudesmol, 0.2 g hairy roots were added 4 mL 70% methanol for 12 h. The extraction was centrifuged for 8 min at 10,000 × *g* to collect the supernatant. After filtering through a 0.22 mm PTFE membrane, the samples were detected by High Performance Liquid Chromatography (HPLC). Atractylodin and *β*-eudesmol chemical molecular standard samples (Sigma) were prepared according to the concentration gradient. Atractylodin and *β*-eudesmol were quantified based on standard curves (Fig. S1 and S2).

After grinding, 0.05 g hairy roots were added 1 mL 70% ethanol for ultrasonic extraction for 2 h at 40 ° C. The Folin-C assay was used to detect total polyphenolic acid (Kupina et al., 2018). The total polyphenolic acid was quantified based on gallic acid standard curves (Fig. S3). The total flavonoid was measured by Aluminum chloride method (Chang et al., 2002), and was quantified based on rutin standard curves (Fig. S4).

2.5 Real time-quantitative PCR (RT-qPCR) analysis and statistical analysis

According to the growth curve of *A. chinensis* hairy roots, hairy roots at 8, 24 and 52 d were collected, and the wild 1-year-old roots of *A. chinensis* were as the control. The key genes involved in the biosynthesis of *β*-eudesmol (*AcOGF30G6*), and genes involved in the biosynthesis of atractylodin (*AcZFP706*, *AcCTA12*, *AcPSPTA25520*, *AcGTF25*) were used for RT-qPCR, and the *Acactin* was as the control gene (Zhang et al. 2024) (Table S1). Total RNA was extracted according to the MiniBEST Universal RNA Extraction Kit (TaKaRa, Beijing, China). First-strand cDNA was performed according to the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Beijing, China). The RT-qPCR reaction system and procedures were referred to the TB Green Premix Ex Taq kit (TaKaRa, Beijing, China). The $2^{-\Delta\Delta t}$ method was used to calculate the relative expression of genes. Each sample has three independent biological replicates. SPSS 23.0 was used to determine the statistical significance ($P < 0.05$).

2.6 Compounds treatments

N-acetyl-5-Methoxytryptamine (melatonin, MT), 5-Methoxytryptamine (5-MT) and 5-Methoxyindole (5-MI) (Solarbio, Beijing, China) were used in this study. The *A. chinensis* hairy roots P-6 were treated with these three compounds. After 52 d of *A. chinensis* hairy roots cultivation, different compounds (MT, 5-MI and

5-MT) with different concentrations (0, 0.01, 0.05, 0.1, 0.2, and 0.5 mmol · L^{-1}) were added to medium to continue cultivating hairy roots. Samples were taken at 168 h to detect the biomass accumulation of hairy roots and contents of *β*-eudesmol, total polyphenolic acid and total flavonoid. Samples were taken at 0, 12, 72 and 168 h for RT-qPCR. Each sample has three independent biological replicates.

3. Results

3.1 The induction and identification of *Atractylodes chinensis* hairy roots

The sterile seedlings of *A. chinensis* preserving in the laboratory were used. The explants were infected by the *Agrobacterium tumefaciens* strains ATCC15834, R1000 and LBA9402, respectively. The induction rates were first calculated, which *A. tumefaciens* strain LBA9402 did not induce *A. chinensis* hairy roots. And induction rate of R1000 was low, being 5%, however, the hairy roots were death quick. Therefore, we focused on the hairy roots induced by ATCC15834, with an induction rate of 85–91% (Table 1). After infection by ATCC15834, hairy roots were observed to grow at the petioles wound site at 20 d in 1/2 MS solid medium used *A. chinensis* petioles. At 80 d, the hairy roots extended and the number of roots increased. And the biomass of *A. chinensis* hairy roots increased extremely at 170 d. On the other side, hairy roots were observed at the leaf wound site at 50 d used *A. chinensis* leaves. At 130 d, the length of hairy roots increased, and branches were observed. And the branches of *A. chinensis* hairy roots increased about 2–3 cm at 170 d (Fig. 1A). To determine the growing hairy roots induced by *A. tumefaciens*, we designed specific primers (*rolB* and *rolC*) and used hairy roots DNA as templates for PCR amplification. The results showed that *rolB* and *rolC* genes were detected in *A. chinensis* hairy roots (Fig. 1B), illustrating that the *A. tumefaciens* Ri plasmid was successfully transferred into *A. chinensis* to induce hairy roots.

Table 1
Different *Agrobacterium tumefaciens* strains induced hairy roots rates of petiole and leaf explants.

A. tumefaciens strains	Petiole			Leaf		
	Number of infections	Number of hairy roots	Induction rate (%)	Number of infections	Number of hairy roots	Induction rate (%)
ATCC15834	34	31	91.2	42	36	85.7
R1000	38	2	5.3	39	2	5.1
LBA9402	35	0	0	40	0	0

3.2 The determination of the optimal medium for growing *A. chinensis* hairy roots

Because the hairy roots grew slowly in 1/2 MS medium, six hairy roots amplification medium with different concentrations of sucrose (3%, 5%) were mixed to screen the optimal medium for growing *A. chinensis* hairy roots. After adding hormones KT with IAA, or NAA, or 6-BA to the 1/2 MS solid medium and culturing with 3% sucrose for 60 d, there were no significant changes. On the contrary, *A. chinensis* hairy roots grew well after adding 5% sucrose for 60 d, with the effect of adding IAA, NAA, and 6-BA to the same concentration of KT on the accumulation of hairy root biomass being IAA > NAA > 6-BA. On the other side, based on the 6,7-V solid medium, adding NAA and 6-BA to the same concentration of KT resulted in higher biomass accumulation in 3% sucrose than in 5% sucrose, and adding 5% sucrose to KT and IAA resulted in higher biomass accumulation than in 3% sucrose (Fig. 2A). According to the above results, our conclusion is that the order of the effect of adding IAA, NAA, and 6-BA to the same concentration of KT at 5% sucrose on the biomass accumulation of *A. chinensis* hairy roots was: IAA > NAA > 6-BA.

Additionally, the biomass of *A. chinensis* hairy roots were also measured to select the optimal medium for growing hairy roots intuitively. The accumulation of biomass in 5% sucrose is generally higher than in 3% sucrose except B2 and B3. And the optimal hormone combination was 0.5 $mg{\cdot}L^{-1}$ IAA + 0.1 $mg{\cdot}L^{-1}$ KT. Finally, *A. chinensis* hairy roots accumulated more biomass in 1/2 MS than in 6,7-V solid medium (Fig. 2B). Our results showed that 1/2 MS solid medium supplemented with 0.5 $mg{\cdot}L^{-1}$ IAA, 0.1 $mg{\cdot}L^{-1}$ KT, and 5% sucrose was the most effective medium combination in increasing the value of hairy roots of *A. chinensis*.

Due to the induction of hairy roots by leaves (L-1) and petioles (P-6 and P-14) of *A. chinensis*. We measured growth curve of these three lines for 52 d, which weighed FW of hairy roots. With the passage of time, the biomass of hairy roots in all three lines increased remarkedly at 52 d (Fig. S5). However, the growth rate of different lines varies. The growth of L-1 was rapid from 8 to 44 d, and tended to stabilize after 44 d. P-6 had two rapid growth phases, including 16–24 d and 48–52 d. And the growth of P-14 was rapid from 0 to 48 d. Overall, among the three lines of hairy roots, the P-6 had the highest biomass accumulation at 52 d, reaching 3.7 g FW. The biomass accumulation of L-1 was the lowest at 52 d, reaching 2.7 g FW. And P-14 was 3.2 g at 52 d. In addition, changes in sucrose concentration in the medium were measured (Fig. S6). There was a significant difference before 24 d of sucrose concentration. After decreasing, there was no significant difference changes from 24 to 36 d. The sucrose concentration decreased from 36 to 40 d, and there was no significant difference in sucrose concentration changes from 40 to 48 d.

3.3 Analysis of metabolites of *A. chinensis* hairy roots

We measured atractylodin, *β*-eudesmol, total polyphenolic acids and total flavonoid of three induced lines to further determine the line with the highest efficiency in inducing metabolites. Firstly, the main metabolites of atractylodin and *β*-eudesmol in *A. chinensis* hairy roots of different lines at different time. Due that the hairy roots on the 0 d were separated from the later stage of the growth cycle, therefore, the hairy roots of 0 d were not included in the comparison of material contents with other time. There were

no significant differences of atractylodin contents of L-1 and P-14. The contents of atractylodin in P-6 at 52 d were significantly higher than other time, reached 1.76 mg·g^{-1} DW (Table 2). In addition, we also compared three lines contents of atractylodin at 52 d with the wild 3-year-old *A. chinensis* (Fig. 3A). The results showed that the content of atractylodin in P-6 was significantly higher than the other two lines, which was no significant difference compared to wild 3-year-old plants.

Table 2
The determination of atractylodin contents of *A. chinensis* hairy roots at different time.

Lines	Atractylodin contents (mg·g^{-1} DW)									
	0 d	8 d	16 d	24 d	32 d	36 d	40 d	44 d	48 d	52 d
L-1	0.54 ± 0.15 a	0.54 ± 0.16 a	0.53 ± 0.21 a	0.37 ± 0.01 a	0.38 ± 0.00 a	0.37 ± 0.01 a	0.38 ± 0.01 a	0.37 ± 0.01 a	0.39 ± 0.02 a	0.37 ± 0.02 a
P-6	1.78 ± 0.08 a	0.40 ± 0.00 c	0.36 ± 0.08 c	0.37 ± 0.01 c	0.95 ± 0.28 b	0.74 ± 0.02 b	0.75 ± 0.02 b	0.78 ± 0.04 b	0.74 ± 0.05 b	1.76 ± 0.08 a
P-14	0.78 ± 0.01 a	0.76 ± 0.02 a	0.75 ± 0.01 a	0.74 ± 0.03 a	0.73 ± 0.04 a	0.73 ± 0.01 a	0.76 ± 0.02 a	0.75 ± 0.01 a	0.53 ± 0.32 a	0.77 ± 0.03 a

Notes: Each value represents the mean ± standard deviation (n = 3). The lower-case letters indicate significant differences among different times via ANOVA followed by Duncan's post-test ($P < 0.05$).

Similarly, we also determined *β*-eudesmol contents. The *β*-eudesmol contents showed a trend of first decreasing and then increasing at 8–52 d, which L-1 and P-6 reached the lowest contents at 32 d, and P-14 reached the lowest contents at 16 d. The contents of *β*-eudesmol in L-1 increased sharply from 32 to 36 d, which reached the maximum at 36 d, and was no significant change at 36–52 d. The contents of *β*-eudesmol in P-6 increased significantly from 36 to 40 d, and was no significant change at 40–52 d. The accumulation of *β*-eudesmol in P-14 was significantly higher at 52 d than other time (Table 3). Therefore, the *β*-eudesmol contents of three lines at 52 d were compared with the wild 3-year-old *A. chinensis* roots (Fig. 3B). Although the *β*-eudesmol contents of hairy roots was significantly lower than control, the P-6 induced by petioles was significantly higher than L-1 induced by leaves.

Table 3
The determination of *β*-eudesmol contents of *A. chinensis* hairy roots at different time.

Lines	*β*-eudesmol contents (mg·g^{-1} DW)									
	0 d	8 d	16 d	24 d	32 d	36 d	40 d	44 d	48 d	52 d
L-1	5.45 ± 0.88 ab	4.69 ± 0.35 ab	5.28 ± 0.57 ab	3.94 ± 1.24 b	3.71 ± 1.02 b	6.09 ± 1.20 a	5.14 ± 0.38 ab	5.02 ± 0.33 ab	4.89 ± 1.01 ab	5.55 ± 0.52 ab
P-6	6.91 ± 0.27ab	4.89 ± 0.04 cd	5.06 ± 0.14cd	4.11 ± 0.33 e	3.45 ± 1.01e	4.04 ± 1.19 e	7.72 ± 0.23 a	7.84 ± 0.23 a	7.12 ± 0.27 ab	7.24 ± 0.26 a
P-14	6.41 ± 0.68 ab	4.96 ± 0.59 bc	3.10 ± 0.36 d	3.71 ± 0.35 cd	4.72 ± 1.03 cd	4.32 ± 0.95 cd	4.71 ± 0.55 bc	5.00 ± 0.06 bc	5.70 ± 0.99 bc	7.10 ± 0.34 a

Notes: Each value represents the mean ± standard deviation (n = 3). The lower-case letters indicate significant differences among different times via ANOVA followed by Duncan's post-test ($P < 0.05$).

Total phenolic acids and total flavonoids were determined and analyzed. The results showed that total phenolic acids of *A. chinensis* hairy roots had no significant change at different time expect L-1 at 8 d and P-14 at 8 and 16 d (Fig.S7 A). And the total flavonoids in P-14 was significantly higher than the other two lines and wild 3-year-old plants (Fig.S7 B). On the other side, the total flavonoids of three *A. chinensis* hairy roots were no significant at different time (Fig. 3C). And the total flavonoids of L-1 were significantly lower than control and other two lines, while P-6 and P-14 at 52 d were no significant difference compared to wild 3-year-old plants (Fig. 3D).

3.4 The expression levels of genes related to metabolites synthesis of *A. chinensis* hairy roots

In order to analyze the ability of *A. chinensis* hairy roots to synthesize metabolites at the molecular level, the key genes involved in the biosynthesis of *β*-eudesmol (*AcOGF30G6*), and genes involved in the biosynthesis of atractylodin (*AcZFP706*, *AcCTA12*, *AcPSPTA25520*, *AcGTF25*) were used for RT-qPCR. The expression of *AcOGF30G6* were significantly up-regulated of three lines at 52 d compared to wild 1-year plants (Fig. 4). The trends of *AcZFP706*, *AcPSPTA25520* and *AcGTF25* increased with the growth of hairy roots in three lines, which *AcPSPTA25520* increased significantly of three lines at 52 d compared to wild 1-year plants. *AcZFP706* in line L-1 and *AcGTF25* in line P-14 increased significantly of three lines at 52 d compared to control. The expression level of *AcCTA12* in line P-14 at 52 d increased significantly compared to control.

3.5 Different concentrations of MT, 5-MI and 5-MT treated to the *A. chinensis* hairy roots

Compared to hairy roots induced from leaves, those induced from petioles exhibited greater biomass accumulation and higher metabolic productivity, particularly for atractylodin and total flavonoids. Among the petiole-derived lines, the P-6 line displayed significantly higher atractylodin content than P-14 and was therefore selected for compound treatments. To investigate the effects of MT, 5-MT, and 5-MI on sesquiterpenoid accumulation in *A. chinensis* hairy roots, the P-6 line was treated with varying concentrations (0, 0.01, 0.05, 0.1, 0.2, and 0.5 mmol·L^{-1}) of each compound. Growth (biomass) and the contents of *β*-eudesmol, total phenolic acids, and total flavonoids were measured. At 168 hours post-treatment (hpt), the biomass of the treated hairy roots showed no significant differences compared to the control (Fig. S8). Analysis of the main sesquiterpenoid metabolite, *β*-eudesmol, revealed that its levels in the hairy roots were significantly lower than in wild 3-year-old *A. chinensis* roots (Fig. 3B). Treatment with 0.5 mmol·L^{-1} 5-MI significantly increased the *β*-eudesmol content to 17.37 mg·g^{-1} DW (Fig. 5A). In contrast, treatments with MT and 5-MT across all concentrations induced no significant changes in *β*-eudesmol levels at 168 hpt. Conversely, treatment with 0.5 mmol·L^{-1} 5-MI significantly decreased the levels of total phenolic acids and total flavonoids (Fig. 5B and C). Treatments with MT and 5-MT at all concentrations resulted in no significant changes in these metabolites at 168 hpt. The increase induced by 5-MI compensated for the initially lower β-eudesmol content in the hairy roots. Specifically, the β-eudesmol level at 168 hpt after 0.5 mmol·L^{-1} 5-MI treatment was significantly higher than that in wild 3-year-old *A. chinensis* roots (Fig. 6). Furthermore, this treatment significantly upregulated the expression of *AcOGF30G6*, a key gene involved in β-eudesmol biosynthesis (Fig. S9).

4. Discussion

The conservations of Traditional Chinese Medicine germplasm resources currently mainly include methods such as seed propagation, artificial cultivation, plant tissue cultivation, and hairy roots cultivation. As an important Traditional Chinese Medicine, *Atractylodes chinensis* (DC.) Koidz. has not been reported to be induced hairy roots, which is unfavorable for its researches and reproduction. Medicinal plants produce a plenty of phytocompounds such as cyclopeptides, quinones, alkaloids, flavonoids and terpenes, which are mainly used for pharmaceutical and flavor industries (Roy, 2021, Miao et al., 2021). Commercial source of these metabolites is field-grown plants, which are generally influenced by seasonal changes and regional differences. Hairy roots culture is considered an alternative method of high-value secondary metabolites for industrial production, which is mass reproduction in a short period of time (Shao et al., 2021). In this study, we established the hairy roots cultivation system of *A. chinensis* efficiently, including determination of the medium, selection of explants and definition of cultivation time.

The establishment of the hairy root cultivation system requires *Agrobacterium rhizogenes*, which contains a special plasmid that induces rooting (Chandra & Chandra, 2011). The efficiency of inducing

hairy roots varies among different strains of *A. rhizogenes*. The strains ATCC15834, R1000, A4, and C58 of *A. rhizogenes* were used to induce hairy roots of *Trigonella foenumgraecum*, which A4 was more conducive to the accumulation of diosgenin in hairy roots than other strains (Zolfaghari et al., 2020). Similarly, A4 strain showed a better transformation frequency compared with ATCC15384, LBA9402, and ACCC10060 to induce *Rubia yunnanensis* hairy roots (Miao et al. 2021). ATCC15834 was found the most efficient strain for *Trachyspermum ammi* L. hairy roots induction (Vamenani et al. 2020). The C58C1 was the best strain for inducing *Atractylodes lancea* hairy roots than A4, K599 and LBA9402 (Zhang et al., 2023a). Other researches indicated that different plants infected by the same strain of *A. rhizogenes*, the induction of hairy roots varies. The induction rates of hairy roots between the *Scutellaria orientalis* and *S. araxensis* infected with ATCC15834, which were 93.3% and 56.6%, respectively (Gharari et al. 2020). In this study, *A. chinensis* hairy roots were successfully induced by *A. rhizogenes* ATCC15834, which the induction rate was highest compared with *A. rhizogenes* R1000 and LBA9402, reaching 85–91%. However, the growth of hairy roots was slow in MS solid medium, which the biomass increased extremely at 170 d. Therefore, it is urgently to optimize the hairy roots culture medium for faster growth.

Previous studies have shown that changing medium composition (Yosephine et al., 2015) or adding exogenous hormones (Putalun et al., 2004) increased the contents of secondary metabolites in hairy roots. Different combinations of exogenous hormones were added to induce different hairy roots. Medium supplemented with 1 $mg{\cdot}L^{-1}$ NAA and 1 $mg{\cdot}L^{-1}$ BA induced *Physalis minima* L. hairy roots efficiently (Putalun et al., 2004). Hairy roots of *A. lancea*, also belonging to the *Asteraceae* crops, were grown on MS0 solid medium supplemented with 3% sucrose, 1.0 $mg{\cdot}L^{-1}$ 6-BA, and 0.1 $mg{\cdot}L^{-1}$ NAA for two months (Zhang et al., 2023a). Therefore, adding exogenous hormones and sucrose were shorten the growth cycle of hairy roots effectively. Sucrose had commonly been used as the carbohydrate source for hairy root growth, and proclaimed superior to other carbohydrate sources such as glucose, mannitol and sorbitol (Banerjee et al., 2012). In this study, we designed 6 combinations of medium with 3% or 5% sucrose to determine the optimal medium for growing *A. chinensis* hairy roots. Combining morphological and biomass growth, 1/2 MS solid medium supplemented with 0.5 $mg{\cdot}L^{-1}$ IAA, 0.1 $mg{\cdot}L^{-1}$ KT, and 5% sucrose was the most effective medium combination.

Hairy roots cultivation has been developed as an innovative approach for the large-scale production of secondary metabolites and plant chemicals, which was cultivated large number of secondary metabolites in a short period of time and continuously supplied high-value products (Korde et al., 2016). And the yield of effective components of secondary metabolites produced by plants takes a long time, which were harvested after 3–5 years of growth to accumulate secondary metabolites a relatively high level (Liu et al., 2019, Ran et al., 2022, Liu et al., 2024). The main active ingredients of *A. chinensis* are volatile oils, eucalyptol and guaiacol sesquiterpenes, and polyacetylenes, which are considered characteristic phytochemicals (Meng et al., 2010). Among them, atractylone, *β*-eudesmol, atractylodinol and atractylodin are main active ingredients (Wang et al., 2023). In this study, we focused on atractylodin and *β*-eudesmol, which possess anti-inflammatory, antibacterial, antiviral and anti-tumor effects (Kimura & Sumiyoshi, 2012, Cheng et al., 2016). And the contents of these two sesquiterpenoids compounds

were similar with Zhang et al. (Zhang et al., 2023b), which the atractylodin contents in hairy roots was significantly higher than normal roots, and *β*-eudesmol contents in hairy roots was lower than normal roots. Moreover, we found that the two lines induced by petioles, especially P-6, produced more metabolites than hairy roots induced by leaves (L-1), including atractylodin, *β*-eudesmol, total polyphenolic acids and total flavonoid. And we were surprised to find that there was no significant difference in the content of atractylodin produced by P-6 compared to the wild 3-year-old plants, reached 1.76 mg/g. Although the *β*-eudesmol of P-6 hairy roots (7.24 mg/g) was significantly lower than control plants (9.53 mg/g) in Inner Mongolia, it was higher than 5-year-old plants (1.61 mg/g) collected in Good Agricultural Practice database, Liuhe City, Changchun, China Changchun City (Liu et al., 2024), which were caused by regional differences. Moreover, the expression levels of genes related to *β*-eudesmol and atractylodin synthesis of *A. chinensis* hairy roots were significantly up-regulated at 52 d compared to wild 1-year plants. These results demonstrated that the contents of secondary metabolites produced by hairy roots that induced by petioles were consistent with wild 3-year-old *A. chinensis*. We estimated profits based on effective content, and the profit of hairy roots was much higher than field planting.

To specifically enhance *β*-eudesmol content in *A. chinensis* hairy roots, this study explored the application of chemical inducers. Melatonin (MT), a well-established inducer, is known to promote the accumulation of various plant secondary metabolites (Wei et al., 2020). For instance, treatment of cabbage with 100 μmol/L MT upregulated anthocyanin biosynthesis genes and stimulated anthocyanin accumulation (Luo et al., 2018). Furthermore, MT-mediated nitric oxide (NO) production has been shown to enhance isoflavone accumulation by boosting phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) activity and upregulating key biosynthetic genes (Yin et al., 2023). However, in our experiments, treatment with varying concentrations of MT failed to significantly induce *β*-eudesmol accumulation in *A. chinensis* hairy roots. We therefore evaluated two chemical homologs of MT, 5-methoxyindole (5-MI) and 5-methoxytryptamine (5-MT). Notably, only treatment with 0.5 mmol·L^{-1} 5-MI resulted in a significant increase in *β*-eudesmol content. This induced level reached 17.37 mg·g^{-1} DW, representing a 1.82-fold increase compared to levels found in wild 3-year-old *A. chinensis* roots. These findings demonstrate that 5-MI acts as an effective inducer for significantly improving *β*-eudesmol content, a key quality marker, in *A. chinensis* hairy roots. This enhancement holds substantial promise for increasing the commercial value and profitability of *A. chinensis* cultivation utilizing hairy root technology.

Declarations

CRediT authorship contribution statement

Z.B. and B.S. conceived the research. J.W., K.W., and X.L. conducted the research. X.L., B.S., and Z.B. analyzed the data. Z.B. and B.S. wrote the manuscript.

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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.

Data availability

The original contributions presented in the study are included in the article/supplementary material.

Ethics declaration: not applicable.

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Figures

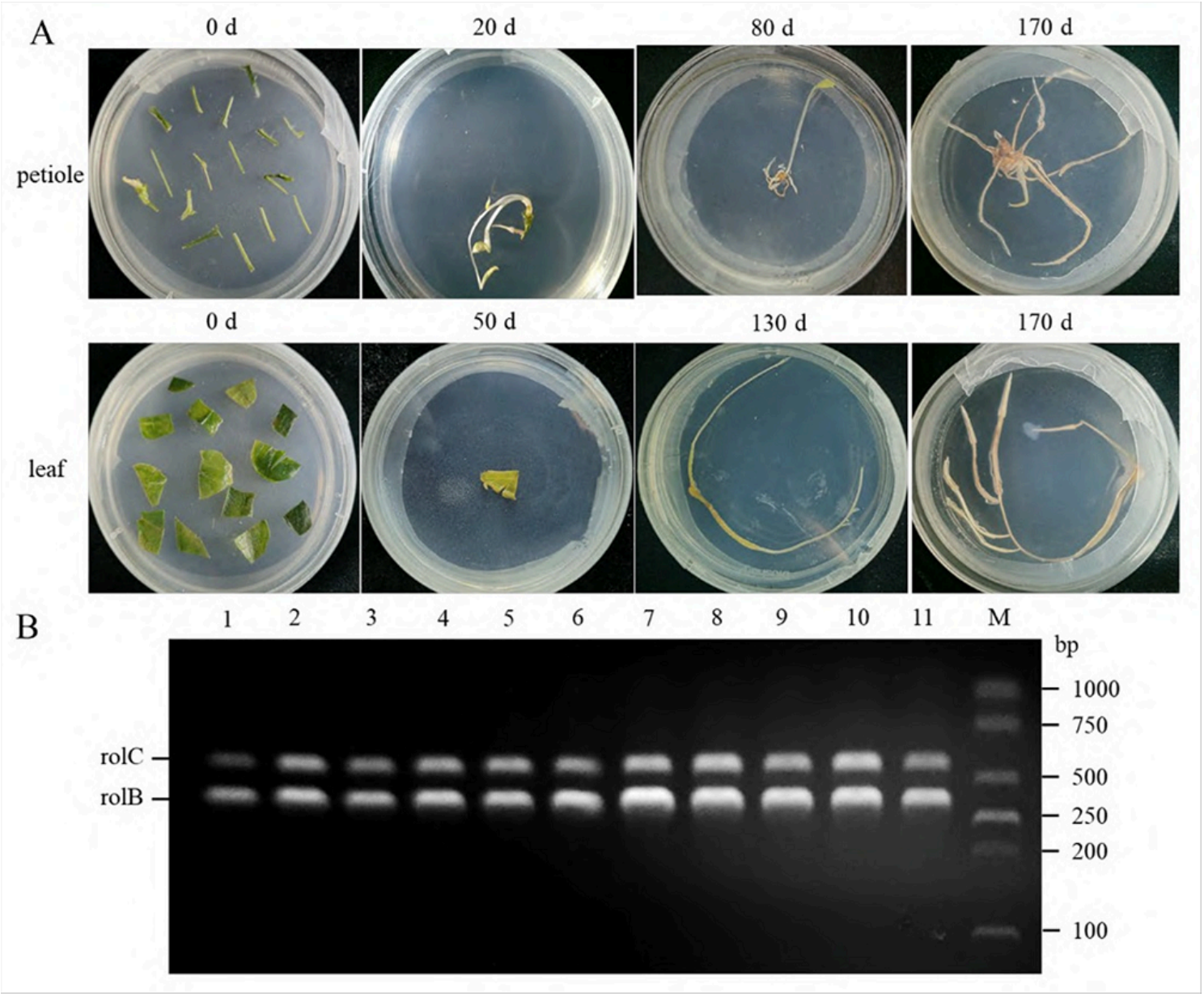


Figure 1

The induction of *Atractylodes chinensis* hairy root. **A**: The hairy roots induced by *A. chinensis* petioles at different time in 1/2 MS medium. **B**: The hairy roots induced by *A. chinensis* leave at different time in 1/2 MS medium. **C**: Identification of *rolB* and *rolC* genes in *A. chinensis*hairy roots.

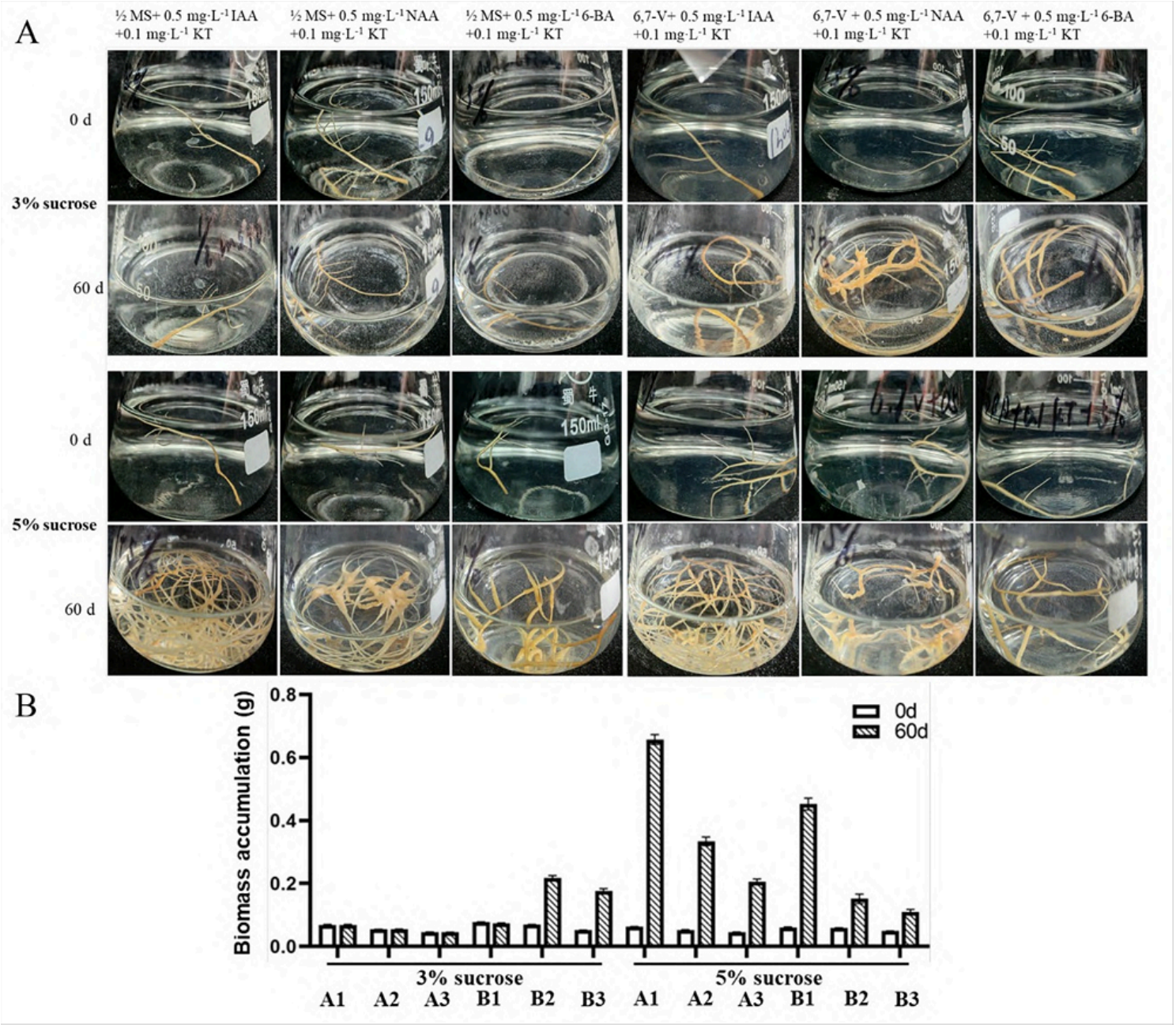


Figure 2

Changes of *A. chinensis* hairy roots in different medium. **A**: The phenotypes of *A. chinensis* hairy roots in different medium. **B**: Biomass accumulation of *A. chinensis* hairy roots in different medium. Each value represents the mean ± standard deviation (n = 3).

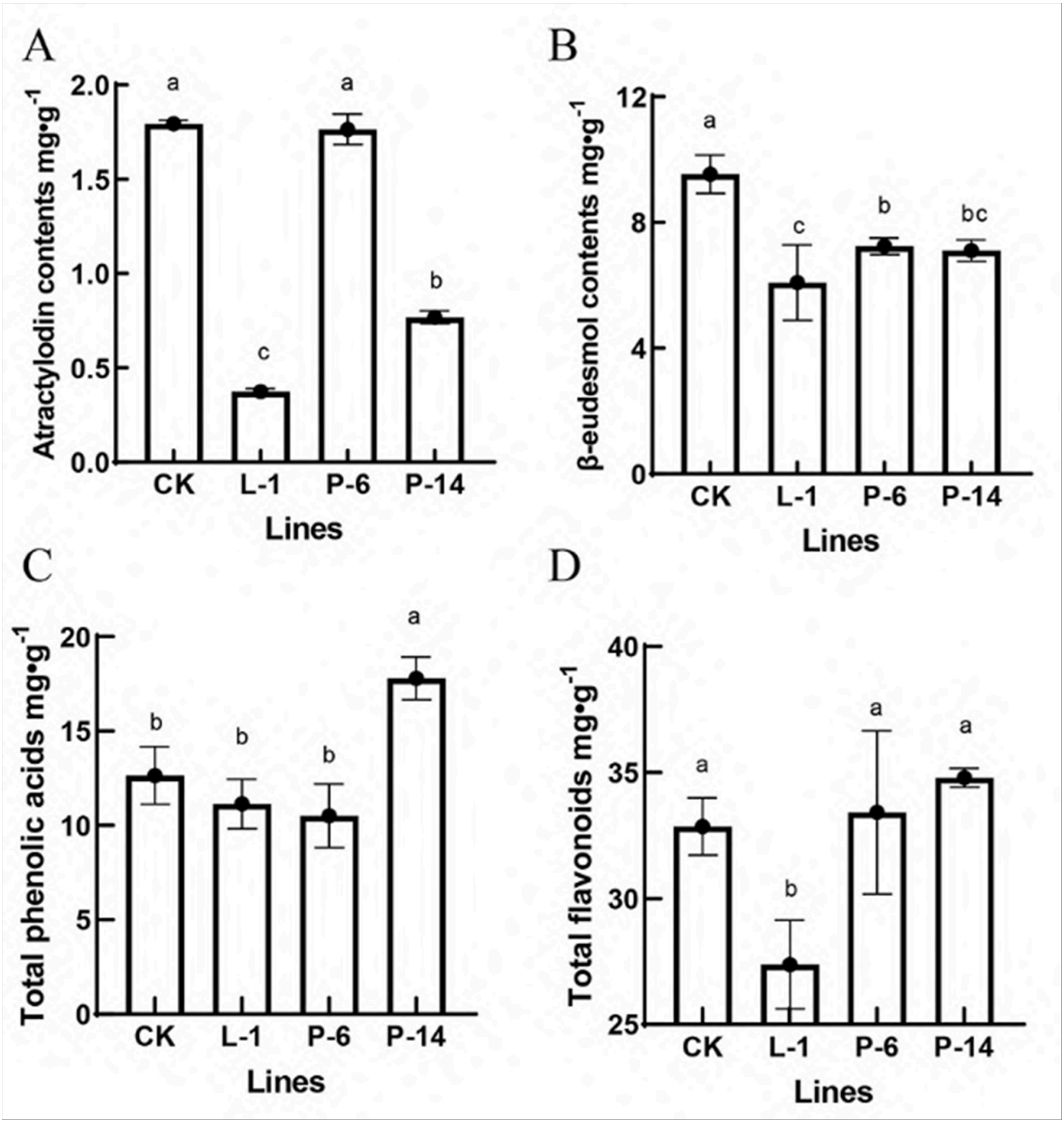


Figure 3

Quantification of metabolites in wild 3-year-old rootsand three hairy roots lines of *A. chinensis*. **A**: Atractylodin contents. **B**: *β*-eudesmol contents. **C**: Total phenolic acids contents. **D**: Total flavonoids contents. Three *A. chinensis* hairy roots lines were collected at 52 d. Each value represents the mean ± standard deviation (n = 3). The lower-case letters indicate significant differences among different lines via ANOVA followed by Duncan's post-test ($P < 0.05$).

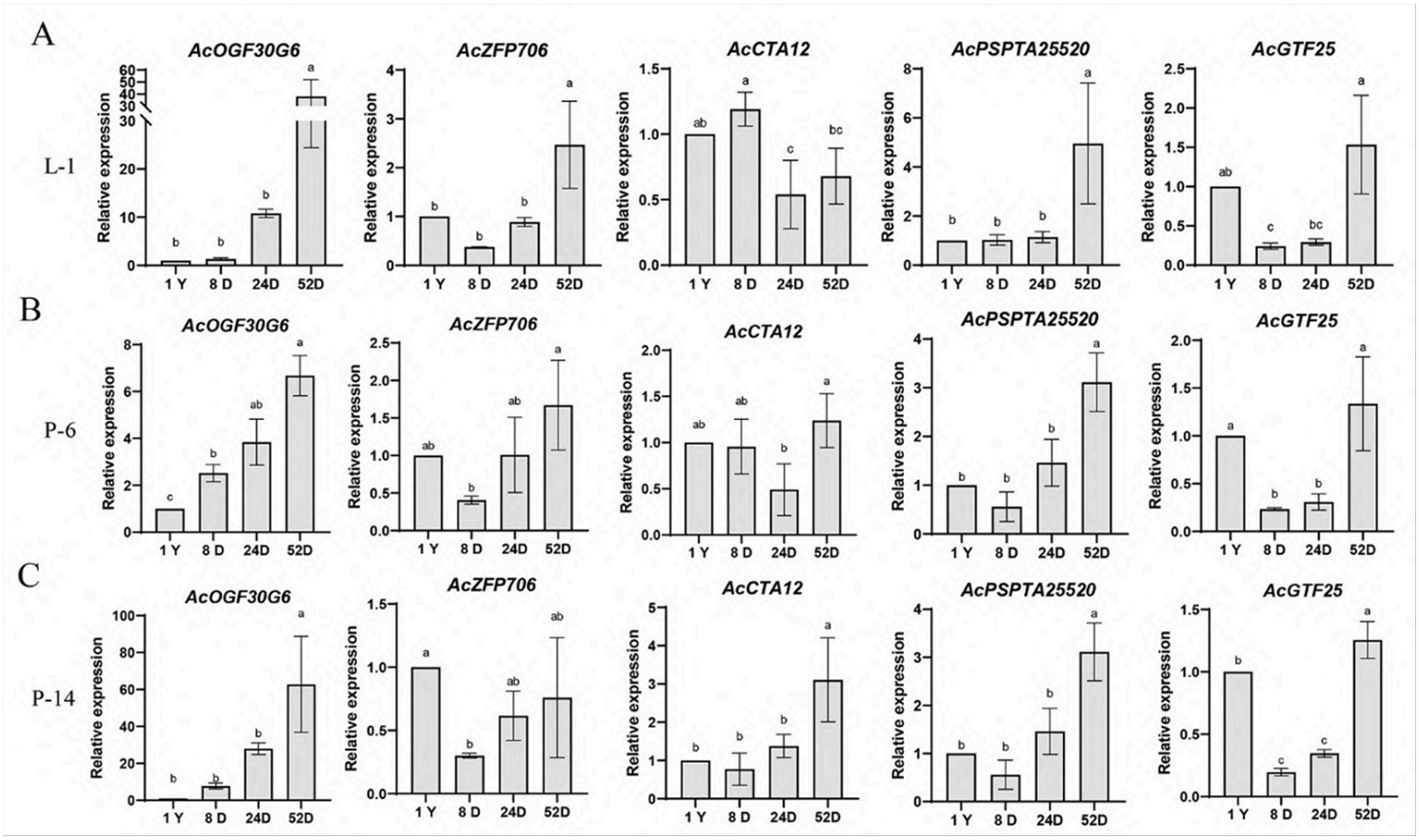


Figure 4

The expression levels of genes related to metabolites synthesis of *A. chinensis* hairy roots. **A**: Lines L-1. **B**: Lines P-6. **C**: Lines P-14. The wild 1-year old roots of *A. chinensis* were as the control. The *AcOGF30G6* gene was involved in the biosynthesis of *β*-eudesmol, and *AcZFP706*, *AcCTA12*, *AcPSPTA25520*, *AcGTF25* genes were involved in the biosynthesis of atractylodin. The lower-case letters indicate significant differences among different lines via ANOVA followed by Duncan's post-test ($P < 0.05$).

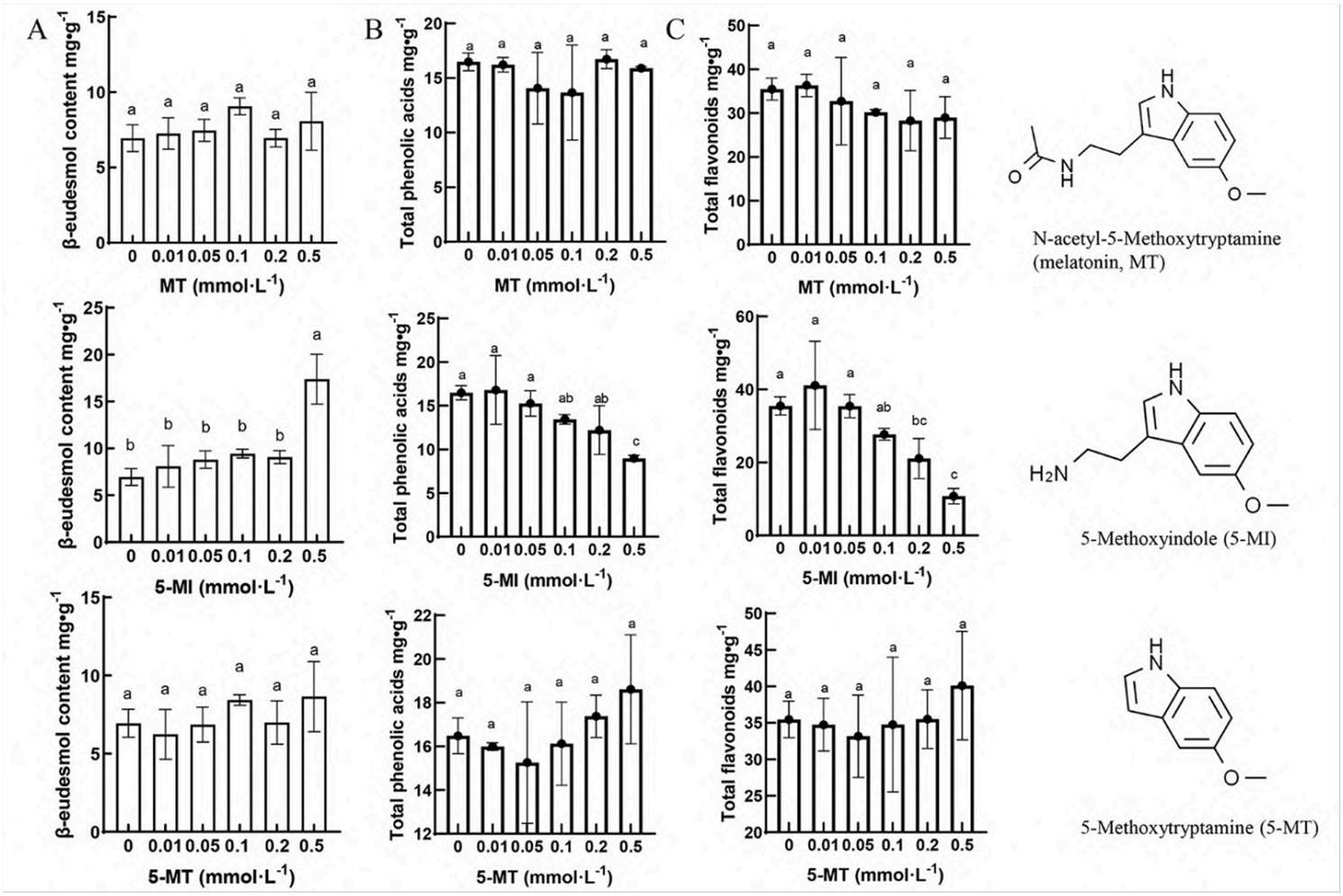


Figure 5

Quantification of metabolites in *A. chinensis* hairy roots after different concentrations of melatonin and its chemical homologs treatments.

A: *β*-eudesmol contents. **B**: Total phenolic acids contents. **C**: Total flavonoids contents. *A. chinensis* hairy roots were collected at 168 hpt. Each value represents the mean ± standard deviation (n = 3). The lower-case letters indicate significant differences among different lines via ANOVA followed by Duncan's post-test ($P < 0.05$).

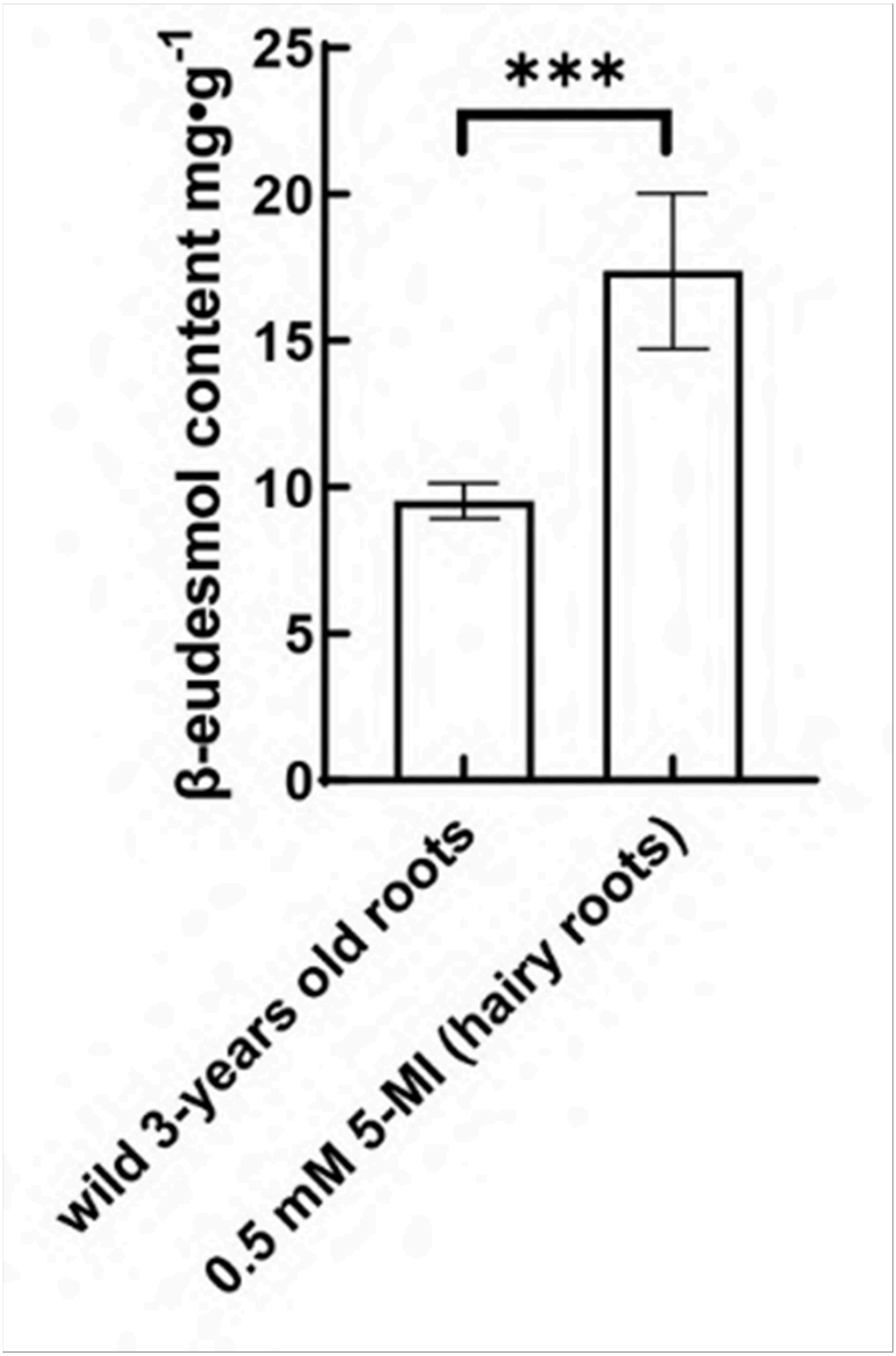


Figure 6

Quantification of *β*-eudesmol contents in wild 3-year-old *A. chinensis* roots and 0.5 mmol·L^{-1} 5-MI treatment *A. chinensis* hairy roots at 168 hpt.

Each value represents the mean ± standard deviation (n = 3). The *** indicate significant differences among different lines via ANOVA followed by Duncan's post-test ($P < 0.001$).

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