Optimal inductive and cultural conditions of *Polygonum multiflorum* transgenic hairy roots mediated with *Agrobacterium rhizogenes* R1601 and an analysis of their anthraquinone constituents

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ABSTRACT

Background: *Polygonum multiflorum* is an important medicinal plant. Hairy roots systems obtained by transforming plant tissues with the natural genetic engineer *Agrobacterium rhizogenes* can produce valuable biological active substances, which have immense potential in the pharmaceutical industry. **Objective:** To optimize the inductive and cultural conditions of *P. multiflorum* hairy roots and to identify the major active secondary metabolites in hairy roots. **Materials and Methods:** *P. multiflorum* hairy root were mediated with *A. rhizogenes* R1601 to induce hairy roots. Four combinations, including Murashige–Skoog (MS), 1/2 MS, B_5, and White, were investigated to optimize the culture medium. MS medium was selected for the growth measurement. The qualitative and quantitative determinations of free anthraquinone in hairy roots were compared with the calli and aseptic plantlets using high-performance liquid chromatography. **Results:** The inductive rates of hairy roots by leaves were higher than for any other explants. The presence of agropine in the *P. multiflorum* hairy roots confirmed that they were indeed transgenic. MS medium was the most suitable of the four media for hairy root growth. Meanwhile, the growth kinetics and nutrient consumption results showed that the hairy roots displayed a sigmoidal growth curve and that their optimal inoculation time was 18-21 days. The determination of the anthraquinone constituents indicated that the rhein content of the hairy roots reached 2.495 $\mu g\ g^{-1}$ and was 2.55-fold higher than that of natural plants. **Conclusion:** Transgenic hairy roots of *P. multiflorum* could be one of the most potent materials for industrial-scale production of bioactive anthraquinone constituents.

Key words: *Agrobacterium rhizogenes* R1601, anthraquinones, *Polygonum multiflorum*, transgenic hairy roots

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INTRODUCTION

Polygonum multiflorum Thunb. (Polygonaceae), better known as "He-Shou-Wu" in the East and "Fo-ti" in the West, is one of the most important and widely used traditional Chinese medicines (TCM) in clinical practice.[1] This herb has been used to treat baldness and hair loss in East Asia, and study results suggest that *P. multiflorum* extract promotes hair growth by inducing the anagen phase in resting hair follicles.[2] It was frequently used as a tonic and purgative in China and Japan.[3] Many reports have discussed its antihyperlipidemic, antioxidation, detoxification, antitumor and intestinal-lubricating bioactivities.[4-9] Many bioactive components, such as anthraquinones, stilbene glycosides, and phenolcarboxylic acids, have been isolated from this plant.[10] The establishment of *P. multiflorum* suspension-cultured cells and their production of useful secondary metabolites were reported by our group.[11]

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Hairy roots systems obtained by transforming plant tissues with the natural genetic engineer *Agrobacterium rhizogenes* (a gram-negative soil bacterium) can produce valuable biological active substances.[12] They can also produce recombinant proteins through the transfer of *Agrobacterium* T-DNA into the plant genome; therefore,

they have immense potential in the pharmaceutical industry.[13,14] Moreover, these genetically transformed hairy roots are capable of unlimited growth in culture media without the need for growth hormones.[15] Their advantages over conventional cell suspension cultures lie in their long-term genetic and biochemical stability in culture.[16,17] The greatest advantage of hairy roots is their greater biosynthetic capacity for secondary metabolite production than that of their parent plants.[18,19] Even in cases where a particular secondary metabolite accumulates only in the aerial part of an intact plant, hairy roots have been shown to accumulate the same metabolite.[20,21] Hairy roots are also known to produce a spectrum of secondary metabolites that are not present in the parent plant.[22] Therefore, the major objective of the present study was to induce, establish, and optimize *P. multiflorum* transgenic hairy roots and then qualitatively and quantitatively identify the major active secondary metabolites (emodin, rhein, and chrysophanol) in hairy roots as compared with the calli and plantlets of this herb using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Plant materials

Polygonum multiflorum shoots were collected from the Medicinal Botanical Garden of Shenyang Pharmaceutical University and identified by Professor Rongmin Yu. The wild shoot explants were cut and gently washed with tap water. Surface sterilization was performed with 75% ethanol for 30 s, and the shoots were then sterilized by soaking in a 0.1% $HgCl_2$ solution for 7 min, followed by washing with sterilized distilled water. Sterile explants were cut to 1 cm^2 pieces and inoculated in Murashige–Skoog (MS)[23] medium for transformation experiments. The inoculated materials were cultivated at 25°C with an illumination period of 16 h light: 8 h dark. Two weeks later, aseptic plantlets were cut from the shoots and cultured under the induction conditions.

Agrobacterium strain

The agropine strain of *A. rhizogenes* R1601 carried the Ri plasmid pRiA_4b containing an *NPT-II* gene inserted into the Hind III fragment 21 of the T_L-DNA and conjugated cosmic pTVK291 containing the part of the *vir* region from the supervirulent Ti plasmid pTiBo542. The *A. rhizogenes* R1601 was maintained on yeast mannitol broth (YMB) medium (1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 5 g L^{-1} peptone, 5 g L^{-1}sucrose, and 2 mmol L^{-1} $MgSO_4$, pH 7.8) containing kanamycin 500 mg L^{-1} at 27°C. Prior to the transformation experiments, the bacterium was sub-cultured in YMB liquid medium and cultured for 48 h in the dark on a rotary shaker (110 rpm) at 27°C. The bacterial suspensions were centrifuged at 5,000 rpm for 25 min and then the pellets were re-suspended in YMB medium.

Callus cultures

For the *P. multiflorum* callus initiation, young leaf segments of aseptic plantlets from the aforementioned plants were taken. The explants were cultivated on MS medium supplemented with NAA (1.0 mg L^{-1}), kinetin (0.1 mg L^{-1}), and sucrose (30 g L^{-1}) at 25°C in the dark. The calli were obtained after 3 weeks and were sub-cultivated on the same medium at 3-week intervals.[24] Callus growth was investigated in 100-mL Erlenmeyer flasks containing 40 mL of the solid MS medium. The inoculum mass of the callus was 1.0 g, and the experimental results were obtained with three replicates.[11,25]

Hairy root induction

Polygonum multiflorum leaves, stems, and leaf stalks of aseptic plantlets from the aforementioned plants were transformed by co-cultivation[26] with *A. rhizogenes* R1601 for 20 min. The infected explants were rinsed three times with sterile distilled water and transferred onto MS medium in an incubator at 25°C in the dark. As a negative control, plant explants were cultured with YMB liquid medium without *A. rhizogenes*. After 2 weeks, individual roots emerging from the wound site were excised and cleared of contaminating bacteria at 37°C on MS medium. Two days later, they were transferred to MS basal medium containing 500 mg L^{-1} cefotaxime at 25°C in the dark. After the bacteria were eliminated, the roots were transferred to MS medium without an antibiotic. The hairy roots were sub-cultured every 2 weeks into fresh medium.

Opine detection in hairy roots

To prove the genetic transformation of the roots, the presence of opines in hairy root cultures was demonstrated as described by Petit.[27]

Hairy roots cultures in different media

Polygonum multiflorum hairy roots used in the experiment were grown in 80 mL of liquid medium in 250-mL Erlenmeyer flasks shaken at 110 rpm in the dark at 25°C. To optimize the culture medium, several combinations were investigated, including MS, 1/2 MS (only the contents of macroelements were half of that in the MS medium, the other elements remained the same), B_5, and White.[15,28] Each flask of cultures was inoculated with 0.3 g of fresh 18-day-old roots. Root growth was assessed by harvesting of the cultures 36 days after inoculation. On each occasion, the hairy roots were separated from the culture medium by filtration. The fresh weights (FW) were determined after they were washed with distilled water and the excess surface water was blotted away. Dry weights (DW) were recorded

after the roots were dried at 50°C in an oven to a constant weight. The growth of the hairy roots was monitored through determination of the harvested FW, DW, and growth ratio (GR) as described by Praveen and Murthy.[29] The GR was determined as the quotient of the DW of the harvested biomass and the DW of the inoculum. Each experiment was performed in triplicate.

Kinetics of growth and nutrient consumption in hairy roots

Based on the experimental results of various media, MS medium was selected for the growth measurements. All experiments were carried out in 250-mL Erlenmeyer flasks containing 80 mL of corresponding medium and inoculated with 0.3 g of fresh weight roots. The hairy roots were incubated on a rotary shaker at 110 rpm and 25°C in the dark. The cultures were harvested and analyzed at 3-day intervals.

FW and DW were measured according to the aforementioned method. The total residual sugar concentration was determined by the method using phenol and concentrated sulfuric acid.[30] The determinations of nitrate, ammonium, and phosphate were achieved using the salicylic acid-concentrated sulfuric acid,[31] phenol-hypochlorite,[32] and ascorbic acid methods, respectively. The pH of the medium was recorded using a Beckman 72 pH meter (Pasadena, California, USA).

Quantitative determination of anthraquinone constituents

Hairy roots, calli and aseptic plantlets were harvested and dried at 50°C in an oven to a constant weight. Dried samples (1 g) of three samples were extracted with chloroform (solid: liquid ratio 1:50) using H_2SO_4 to soak previously in an ultrasonic bath (3 × 20 min). The extracts were combined, evaporated to dryness and re-dissolved in methanol (5 mL). The samples were filtered through a 0.45-μm filter membrane and then subjected to HPLC analysis.[33] The anthraquinone fractions were analyzed using Agilent 1100 HPLC (Palo Alto, California, USA) equipped with diode-array detection (HPLC-DAD) and an SB-C_{18} column (5 μm, 4.6 × 250 mm). The mobile phase was a mixture of methanol and 0.2% H_3PO_4 (82:18, v/v) at a flow rate of 0.9 mL $min^{-1}$and column temperature was maintained at 30°C. The detection wavelength was set at 289 nm. The injection volume was 20 μL. The chromatography system was equilibrated according to the mobile phase. The standard samples of rhein, emodin, and chrysophanol were purchased from the National Institutes for Food and Drug Control of China. The purity was above 98% determined by HPLC.

Statistical analysis of the data

All experiments were repeated in triplicate. Data from the shake-flask cultures were averaged from three independent experiments with two biological replicates. HPLC-DAD quantitative measurements were done in triplicate. Statistical calculations by SPSS software were conducted to calculate the correlation. Values are presented as means ± SD (n = 3).

RESULTS AND DISCUSSION

Hairy root induction

Different parts (leaves, stems, and leaf stalks) from aseptic plantlets of *P. multiflorum* were mediated by co-cultivation with *A. rhizogenes* strain R1601. Five days later, hairy roots emerged from the leaf veins and displayed a white and more hairy phenotype. Hairy roots appeared from underneath the leaf stalks and stems after 10-day of culturing.

Leaves, stems, and leaf stalks showed different transformation rates. The leaves had the highest infection rates among the three (up to 41.67 ± 15.82%), and the roots showed typical morphology of thick lateral branching, plagiotropic root growth, and root hair profusion. The inductive rate of the leaf stalks was 12.50 ± 5.92%, while that of the stems was about 15%. It is possible that the tender organ was susceptible to the mediation with *A. rhizogenes* R1601 to induce the hairy roots. In the meantime, all hairy roots from the different explants displayed characteristics of rapid growth. As a negative control, untransformed adventitious roots turned up from *P. multiflorum* leaves were co-cultured with YMB liquid medium without a strain and had a different phenotype from the hairy roots, such as being thinner, white-yellow, fragile, and having no lateral branches.

The optimal hairy roots were screened out on hormone-free MS medium for their fast growth, abundant lateral branching after bacterial elimination at 37°C for 2 days, and antibiotic. *P. multiflorum* hairy roots excised from the parent explants and transferred to new medium for several subcultures were selected and used for complete and stable genetic transformation assays, growth kinetic studies, nutrient consumption, and anthraquinone constituent analysis.

Opine detection in the hairy roots

To investigate whether transformation with an Ri plasmid (pRiA4b) had occurred, the presence of opines was examined using paper electrophoresis and silver nitrate staining. It was clearly demonstrated that T_R-DNA was integrated into plant genome due to the detected agropine. The T-DNA localization in the plant genome serves as a reliable genetic marker for confirming the transformation.[22] This result demonstrated that the hairy roots were indeed transgenic.

Basic medium selection of hairy root culture

Four different media (MS, 1/2MS, B_5, and White) were tested to optimize the culture conditions. Excised hairy roots grow slowly until 12 days of culture. After another 6 days, the hairy roots grew to 5.0 cm long. The hairy roots were harvested on day 36 of culture, and the growth parameters were calculated [Figure 1].

The growth rate of hairy roots on MS medium was significantly higher than that on other media, up to 19.30-fold, whereas B_5 and White media were not suitable for the hairy root culture under the experimental conditions [Figure 1]. Thus, MS medium containing higher hyper-saline concentrations and nutrients was the best basic medium for the *P. multiflorum* hairy roots.

Growth kinetics and nutrient consumption of hairy roots

After mediating the *P. multiflorum* hairy root cultures and determining the growth as described above, we established the growth curve [Figure 2]. The hairy roots displayed a relatively slow S-shaped growth curve, its lag phase was very apparent until the fifth sampling time on day 15, and the stationary phase began on day 18. The average GR was 19.3 (DW) and their doubling time was approximately 6 days. The maximum fresh and dried growth rates of the hairy roots were 10.44-fold and 19.3-fold. The result indicated that less inoculum results in lower biomass increase. These data suggest that the optimal sub-culture time was 18-21 days of culture.

The pH of the medium rose slightly from the start of culture to day 5 and then dropped until day 25, after that, it began to increase again. During the incubation period, the pH value of the medium fluctuated between 3.8 and 5.3. The result demonstrated that MS medium has a strong buffering capacity and could supply adequate nutrition for hairy root growth.

Residual sugar and phosphate were detected during the culture period [Figure 3]. The sugar was utilized slowly during the culture period. The biomass accumulation [Figure 2] was associated with the carbohydrate concentration in the medium, and biomass increases were associated with total sugar removal. Our findings suggested that the absorbed carbohydrates were used for the primary metabolism. The phosphate was almost depleted within the first 30 days of culture, whereas the phosphate tended to leak from the hairy roots into the medium in the latter part of the growth cycle. This was possibly related to the fact that hairy roots need adequate phosphate for rapid growth, and the phosphate leaking into the medium might be attributed to phosphate self-release when it was synthesized by inorganic elements as a function of acid phosphatase.[34]

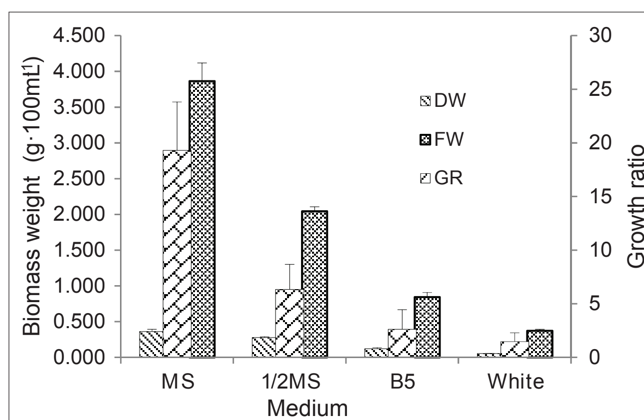

Figure 1: Effects of four media on the growth of transgenic hairy roots. Hairy roots (0.3 g) were cultured in 250-mL Erlenmeyer flasks containing 80 mL of Murashige-Skoog medium for 36 days. Data represent mean values ± SE of three replicates

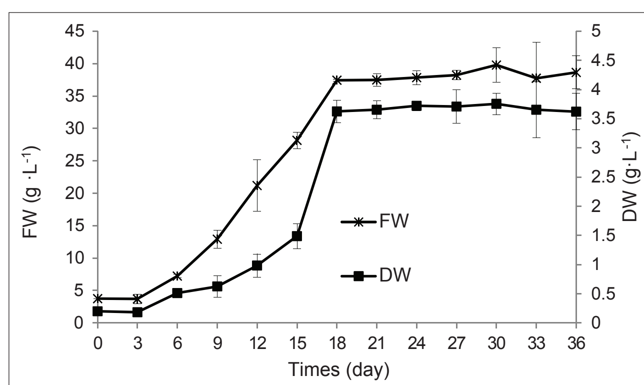

Figure 2: Growth curves of hairy roots of *Polygonum multiflorum*

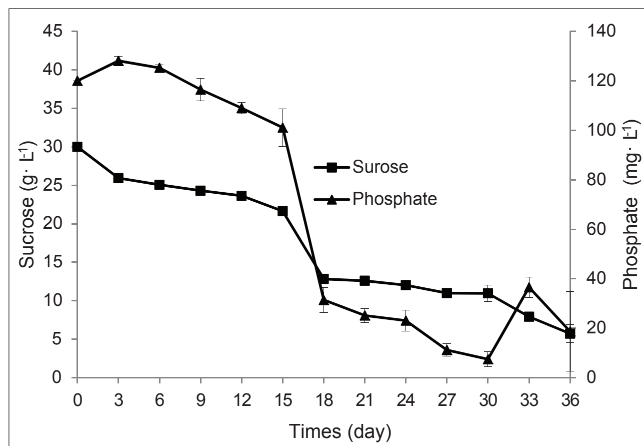

Figure 3: Time course of residual sugar and phosphate uptake in hairy roots of *Polygonum multiflorum*

The results of nitrate and ammonium uptake during the culture cycle are shown in Figure 4. Nitrate uptake was higher than that of ammonium from the beginning of culture to day 12 and from day 24 to day 30. It was possible that the fast ammonium uptake supported protein synthesis for the hairy root growth and that the nitrate utilization was related to the activation of nitrate reductase and nitrite reductase.

In conclusion, this investigation of the growth and nutrient consumption kinetics was beneficial for evaluating the cultural course and metabolic regulation and will be valuable for the production of bioactive compounds in industrial-scale cultures by bioreactors.

Anthraquinone analysis

Free anthraquinone fractions were extracted from hairy roots, calli, and aseptic plantlets and then analyzed using HPLC [Table 1]. The rhein content reached 2.495 μg g^{-1} in the hairy roots and was 2.55-fold higher than that in the natural plant. The emodin content in the hairy root reached 87.631 μg g^{-1} and was only 29.0% of that in the parent plant, whereas its level was still 210.1 times higher than that in the calli. Chrysophanol content was fairly low in the hairy roots, whereas it could not be detected in the callus or natural plant; therefore, its content in hairy roots was higher than that in the other two materials.

Polygonum multiflorum hairy roots might be attractive industrial materials in the production of bioactive anthraquinones. We inferred that the content of anthraquinone compounds would increase if some external conditions changed or special factors and genes were administered (e.g., biotic elicitor, abiotic elicitor, *rolC* genes).[29] These technologies would help enhance the anthraquinone content of *P. multiflorum* hairy roots.

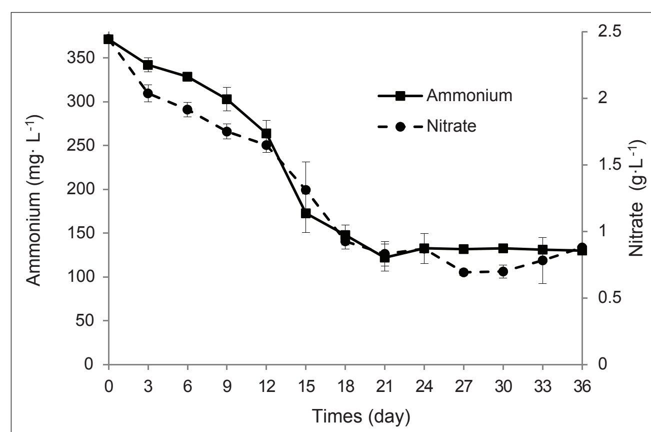

Figure 4: Time course of uptake of nitrate and ammonium in hairy roots of *Polygonum multiflorum*

Table 1: Anthraquinone contents in *Polygonum multiflorum* materials (in μg g^{-1} DW) detected using HPLC

Material	Emodin	Rhein	Chrysophanol
Hairy root	87.631±0.103	2.495±0.022	Trace
Natural plant	302.102±0.127	0.979±0.049	-
Callus	0.417±0.0003	3.835±0.089	-

DW: Dry weight; HPLC: High-performance liquid chromatography

CONCLUSIONS

Hairy root cultivation is an efficient method for producing useful phytomolecules on an industrial scale. The results of the present study demonstrated that *P. multiflorum* leaves had the highest induction rate when different explants were co-cultivated with *A. rhizogenes* strain R1601. The optimal culture of hairy roots was then screened out, and MS medium was found to be the most suitable medium for *P. multiflorum* hairy roots. Most vigorous hairy roots showed stable growth under submerged cultivation and accumulated high biomass amounts. Furthermore, the growth curve and nutrient consumption of *P. multiflorum* hairy roots during the culture period were also established and investigated. More importantly, free anthraquinone analysis using HPLC showed that the rhein content in *P. multiflorum* hairy roots was 2.55 times higher than that in the natural plants. Therefore, transgenic hairy roots of *P. multiflorum* could be one of the most potent materials for industrial-scale production of bioactive anthraquinone constituents.

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