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ORIGINAL ARTICLE

In vitro production and distribution of flavonoids in *Glycyrrhiza uralensis* Fisch.

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Abstract *Glycyrrhiza uralensis* Fisch. is known as a common Chinese medicinal herb used to harmonize the effects of other ingredients in most Chinese herbal prescriptions. The rapid production of flavonoids in vitro remains unknown in *G. uralensis* Fisch. To investigate the in vitro adventitious root regeneration and flavonoid accumulation characteristics in *G. uralensis* for restrictions on collecting wild plants, suspension cultural and freezing microtomy with histochemical assays were carried out. We reported that multiple adventitious roots were initiated from hypocotyls and stems of *G. uralensis*. Indole-3-butyric acid (IBA) was more conducive than NAA (1-naphthaleneacetic acid) in inducing *G. uralensis* adventitious roots, but the addition of 6-BA (6-benzylaminopurine) and KT (kinetin) suppressed the formation of adventitious roots. While the concentration of IBA was 1.0 mg L^{-1}, the flavonoid content and yield were the highest at 19.96 mg g^{-1} and 1.23 mg g^{-1}, respectively. The optimum medium for adventitious root induction was 1/4-strength Murashige and Skoog's medium containing 0.1 mg L^{-1} IBA. The content of flavonoids in adventitious roots and apicals cultured in vitro was higher than that in suspension callus, reaching 3.87 times the callus flavonoid content. The histochemical localization of flavonoids showed that *G. uralensis* flavonoids mainly distributed in the epidermal parenchyma cells of the callus outer layers and gradually accumulated in cell wall and cell gaps of the epidermis and endodermis of adventitious roots along with the primary growth of adventitious roots, indicating that there were no flavonoids in the roots at the early stage of adventitious roots formation. The results showed that calli inducing adventitious roots and apicals for 30 days obtained the highest yield of flavonoid, indicating effective production for flavonoids instead of wild culture. $AlCl_3$ ethanol solution was better than NaOH aqueous solution in terms of chromogenic and localization effects. We concluded that the highest yield of flavonoid and effective production for flavonoid instead of wild culture could be obtained from calli inducing adventitious roots and apicals.

Keywords *Glycyrrhiza uralensis* Fisch. · Suspension culture · Adventitious root · Flavonoids · Histochemical localization

Abbreviations

IBA	Indole-3-butyric acid
NAA	1-Naphthlcetic acid
6-BA	6-Benzylaminopurine
KT	Kinetin
MS	Murashige and Skoog
F	Flavanone liquiritigenin
C	2'-Hydroxychalcone isomer, isoliquiritigenin
CHI	Chalcone isomerase gene
CHS	Chalcone synthase gene
C4H	Cinnamic acid-4-hydroxylase

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Introduction

With a 3000-year history of being used as a medicinal plant, *Glycyrrhiza uralensis* Fisch. is known as one of the most ancient herbal medicines (Zhang et al. 2009). *G. uralensis* inflata is a common Chinese medicinal herb used to harmonize the effects of the other ingredients in most Chinese herbal prescriptions (Yang et al. 2009). The main effective constituents are flavonoids and saponins (Yang et al. 2008). Many scholars have isolated and identified 61 kinds of triterpenoid saponins and more than 300 kinds of flavonoids from licorice (Fukai et al. 2002; Shul'ts et al. 2000). Furthermore, licorice extracts are used in cosmetics, food additives, tobacco flavors and confectionery foods (Hayashi and Sudo 2009). Many biological activities, such as anti-mutagenic activity, anti-ulcer effects, protective action against hepatotoxicity, antitumor-promoting activity and antimicrobial effects, have been reported (Li et al. 2000). Due to the decrease of wild *Glycyrrhiza* resources and restrictions on collecting wild *Glycyrrhiza* plants, researchers have attempted to enhance flavonoid accumulation in licorice cells and search for substitutes for licorice biotic resources by means of biotechnology (Yang et al. 2006). The flavanone liquiritigenin (F) and its 2'-hydroxychalcone isomer, isoliquiritigenin (C) compositions and proportions were found to be constant for all extracts from a *Glycyrrhiza species*. All *G. uralensis* extracts contained up to 2.5 more flavanone liquiritigenin than *G. glabra* extracts (Simmler et al. 2014).

In recent years, with the development of molecular biology, the use of genetic modification and genetic engineering to regulate the production of medicinal plant secondary metabolites has become a research hotspot, effectively alleviating the shortage of Chinese herbal medicine resources, and has become an effective way to improve secondary metabolites. For example, hairy root cultures of *Saussurea involucrata* have been transformed with the chalcone isomerase gene (CHI), producing more flavonoids than wild-type hairy roots (Li et al. 2006). In contrast, the silencing of the chalcone synthase gene (CHS) in transgenic hairy roots of *Medicago truncatula* decreased flavonoid production (Wasson et al. 2006). Zhang et al. (2009) significantly increased the licorice flavonoid yield in hairy roots through over-expression of CHI. They considered that different elicitors combined with the overexpression of the synthase gene can promote the accumulation of flavonoids in licorice more effectively. The overexpression of CHI promotes the yield of flavonoids in the hairy roots of *Scutellaria baicalensis* (Park et al. 2011). CHS and other synthetic genes have a synergistic expression effect (Tunen et al. 1988). Cinnamic acid-4-hydroxylase (C4H) is the second key enzyme of the phenylpropanoid pathway, affecting the metabolism of flavonoid synthesis. Studies showed the expression of C4H had a direct impact on the flavonoid content of *Rubus triflorus* and *Camellia sinensis* (Baek et al. 2008; Singh et al. 2009). The production of licorice chalcones and total flavonoids of hairy roots were improved by adding Tween-80, and increased flavonoids had a relationship with mRNA levels, the activities of phenylalanine ammonialyase, 4-coumarate-coenzyme a ligase and cinnamate-4-hydroxylase (Zhang et al. 2011). Jiang et al. (2010) used RNAi from *Glycine max* (L.) Merr. isoflavone synthase gene to promote the accumulation of isoflavones.

Plant in vitro culture is an alternative source for the production of valuable secondary metabolites. Hairy root cultures are valuable sources of medicinal compounds. The interest in hairy roots be due to their ability to grow fast without an external supply of any plant growth regulator. Many studies on in vitro culture of licorice, including callus, suspension, hairy roots and whole plant cultures, have been reported (Arias-Castro et al. 1993; Ayabe et al. 1990; Hayashi et al. 1988; Shabani et al. 2009; Wang et al. 2010; Wongwicha et al. 2008; Yang et al. 2007). Since Kamada et al. (1986) established the hairy root culture system using Agrobacterium to infect *Atropa belladonna*, culturing medicinal plant hairy roots to obtain secondary metabolites has become a new research hot spot; it is also considered to be an effective method to obtain secondary metabolites followed by cell culturing. Liquid culture systems with elicitors are increasingly being investigated to improve secondary metabolite production and to reduce processing costs in several plant cell/hairy root cultivation systems (Prakash and Srivastava 2008). A number of studies showed that the type and age of explant have a strong influence on hairy root induction since the age of the explant is a major factor that alters the physiological properties of the cell (Dupre et al. 2000). Hairy root cultures are characterized by high biosynthetic capacity and genetic as well as biochemical stability; therefore, they are considered to offer better prospects for the commercial production of secondary metabolites than undifferentiated cell cultures (Toivonen 1993). The effect of Tween 80 as an elicitor of licochalcone A from hairy root cultures of *G. uralensis* has been evaluated. After a 15-day treatment with 2% Tween 80, hairy roots grew well and produced higher levels of licochalcone A and total flavonoids than the control (Zhang et al. 2011). Wongwicha et al. (2011) induced hairy roots with licorice leaves, and stems grew fast in 1/2 MS medium.

Adventitious root induction is influenced by various factors, including genotype, plant growth regulators, other medium components and culture conditions (Liu et al. 2010; Zhu et al. 2010; Reis et al. 2011). Awad et al. (2011) considered that MS medium with 3% sucrose was

suitable for licorice apical in vitro culturing, and the licorice acid content was 1.32 mg g^{-1}. In order to protect wild licorice resources, organ and tissue in vitro culture is a very effective way of obtaining licorice secondary metabolites (Karuppusamy 2009). Currently, studies on licorice products obtained using tissue culture are more concentrated on cell and hairy root culturing, while studies on culturing licorice adventitious root to effectively obtain secondary metabolites are limited. Cells and root tips of *G. uralensis* were used to induce adventitious roots to acquire secondary metabolites of licorice.

Materials and methods

Plant explants and treatments

Glycyrrhiza uralensis Fisch. seeds were collected from Province Neimenggu, China, in the middle of May 2017. The seeds were stripped and sterilized with 4% sodium hypochlorite solution for 10 min and then rinsed four times with sterile water. After sterilization, explants were placed in 100-mL flasks containing several media. They were incubated at a 22/20 °C thermoperiod (light/dark) under a 17-h photoperiod (40 μmol m^{-2} s^{-1}, cool white fluorescent tubes).

Callus suspension culture

The hypocotyl of *G. uralensis* was inoculated on a medium of MS + 6-BA1.0 mg L^{-1} + NAA 2.0 mg L^{-1} for inducing callus. Then, 1.0 g callus was inoculated on a liquid medium of MS + 2,4-D 0.2 mg L^{-1} + 6-BA 0.5 mg L^{-1} + NAA 0.5 mg L^{-1} (pH 5.8), and the results for the remaining 15 liquid media are shown in supplementary Table S1 and Fig. S1. Callus were incubated at 25 °C on a rotary shaker (115 rpm) in darkness, subcultured 2–3 times in liquid medium every 10 days, and the dry weight, content and yield of flavonoids in callus were measured every 3 days. A 30-day growth curve of callus was drawn. Each treatment was repeated three times.

Adventitious root induction from callus

Callus (0.5 g) was inoculated on 1/2 MS culture solution; there was a total of seven media with different hormone combinations. Callus were incubated at 25 °C on a rotary shaker (115 rpm) in darkness. Each treatment was repeated three times. The induction of callus growth and adventitious roots were observed every day, and the fresh weight, dry weight and flavonoid accumulation of the callus were determined 30 days later.

Adventitious root induction from the stem

The stem of *G. uralensis* was inoculated on a medium of 1/2 MS + IBA (0.5 mg L^{-1}) to induce adventitious roots. Seven days later, about 0.2 g of 1 cm-long adventitious root tips was clipped and inoculated on a total of seven media. They were incubated at 25 °C on a rotary shaker (115 rpm) in darkness for 3 weeks, and then the fresh weight, root length, number of lateral roots, content and yield of flavonoids were measured.

The standard sample

A 2.5 mg glycyrrhizin standard sample was dissolved in methanol as a control solution of 0.1 mg mL^{-1}. Then, 0.2 mL control solution was added to methanol to a volume of 1 mL, and 1 mL of 10% KOH was added 5 min later, after which methanol was added to a volume of 5 mL. Methanol was used as a blank control for scanning under a 200–500 nm wavelength. Glycyrrhizin standard solution has a maximum absorption value at 336 nm (Fig. S2).

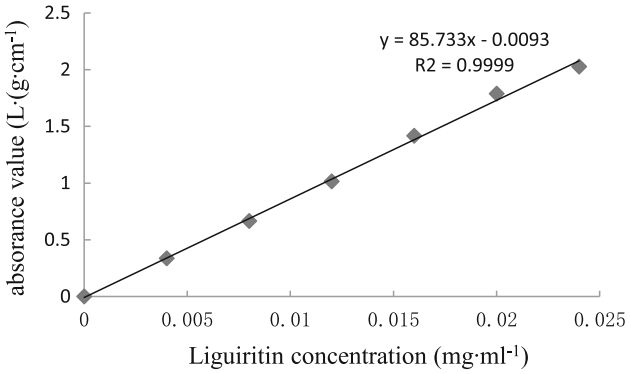
The method was as follows: place 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mL of the control solution into 10 mL centrifuge tubes; set the volume to 1.5 mL with methanol; add 1 mL 10% KOH solution. Shake centrifuge tubes and allow color development for 5 min; set the volume to 5 mL with methanol, and measure absorbance values at 336 nm. Standard curves were drawn using the reference concentration as the abscissa and the absorbance value as the vertical axis.

Glycyrrhizin standard sample concentration and absorbency

liquiritin concentration (mg mL^{-1})	0	0.004	0.008	0.012	0.016	0.02	0.024
absorbance value (L g cm^{-1})	0	0.333	0.663	1.011	1.366	1.709	2.069

Standard curve of liquiritin

The regression equation of glycyrrhizin: $y = 86.268x - 0.0136$ ($R^2 = 0.9998$), where x is the glycyrrhizin concentration of the colored solution, and y is the absorbance of the colored solution at 336 nm.



Extraction and measurement of flavonoids

Callus was put into Petri dishes for drying to a constant weight at 60 °C. Callus (0.1 g (± 0.002 g) was ground, sieved, and then put into centrifuge tubes. Ethanol (5 mL, 75%) was added to the centrifuge tubes, followed by shaking and soaking for 1 h. Ultrasonic wave were used - to break the cell for 40 min, then centrifuged for 10 min (7104×*g*). The supernatant was used for the test sample solution: draw 0.2 mL of sample solution and add 0.8 mL of methanol and 1 mL of 10% KOH solution. Five minutes later, add methanol to 5 mL, and measure absorbance at 336 nm (See supplemental Fig. S2). Each sample was repeated three times. The total flavonoids of samples were calculated according to the standard curve. Flavonoid content formula:

$$\text{Flavonoid content}\,(\text{mg g}^{-1}) = \text{C} \times \text{N} \times \text{V/W} = \text{C} \times 25 \times 5\,\text{mL}\,0.1\,\text{g}^{-1}.$$

The flavonoid content was calculated as glycyrrhizin. C is the concentration of flavonoids in the colored solution (mg mL^{-1}), N is the dilution factor, V is the volume of extraction of flavonoids (5 mL), W is the weight of callus (0.1 g):

$$\text{The flavonoid yield}\,(\text{mg}) = \text{callus dry weight}\,(\text{g}) \times \text{flavonoid content}\,(\text{mg g}^{-1}).$$

Histochemical localization of flavonoids

In order to identify the space distribution character of flavonoids in callus and adventitious roots, we observed freezing sections of fresh callus and adventitious roots. Samples were frozen, and 30-μm-thick sections were serially produced by a Leica CM1950 freezing microtome (Leica Instruments, Shanghai, China). The sections were stained with 10% NaOH and 1% $AlCl_3$ ethanol solution for 20 min and were then mounted using glycerol / water (15:85). The sections were observed with a Leica DM 2500 light microscope (Germany) and a Leica DM 6000B fluorescence microscope (Germany) immediately. The location of flavonoids in *G. uralensis* was observed through two different staining methods.

Results and discussion

Callus suspension culture

The growth curve of the suspension callus was S-shaped (Fig. 1a). The first 3 days of cell growth was a lag phase, and the cells grew very slowly, then quickly entered the logarithmic growth phase; the callus consisted of yellow grains. The culture solution became turbid on the 9th day, and cellular metabolic waste attached to the sides of the bottles gradually. The stable phase of cell growth began on the 21st day, and the cell dry weight almost stopped increasing, which was about 7.5 times of the initial dry weight. Twenty-four days later, the cell dry weight decreased significantly, and cell color changed from yellow brown to dark brown. Licorice callus cells in good condition on the 18th to 21st days were selected for subculturing.

In the suspension culture process, the *G. uralensis* flavonoid content in callus cells increased first and then decreased (Fig. 1a). The initial content of flavonoids was 6.853 mg g^{-1}. The highest content of 13.471 mg g^{-1} appeared on the third day, and it was 1.966 times higher than the initial content, probably resulting from the adaptation period in the new environment and the rapid generation of a stress response of flavonoids to prevent cell damage. The flavonoid content of the logarithmic growth phase cells decreased gradually and decreased rapidly on the sixth day, being slightly higher than the initial content of flavonoids, i.e., 8.166 mg g^{-1}. Then, on the 15th day, there was a clear downward trend. Twenty-one days later the trend stabilized, but the content of flavonoids was 5.156 mg g^{-1} lower than the initial content. Therefore, the accumulation of flavonoids was associated with the cell growth rate. During suspension culture, flavonoid yield was proportional to the dry weight of cells (Fig. 1a). With culturing duration, the yield of flavonoids increased gradually. On the 21st day, the yield of flavonoids (3.818 mg g^{-1}) increased to 6.332 times of the initial yield (0.603 mg g^{-1}).

Adventitious root induction in callus

The effect of hormone combinations on the induction of adventitious roots

The effects of the NAA, IBA, 6-BA and KT combinations on the induction of adventitious roots in callus were studied. NAA and IBA either alone or with KT or 6-BA could induce suspension callus, forming adventitious roots. In all

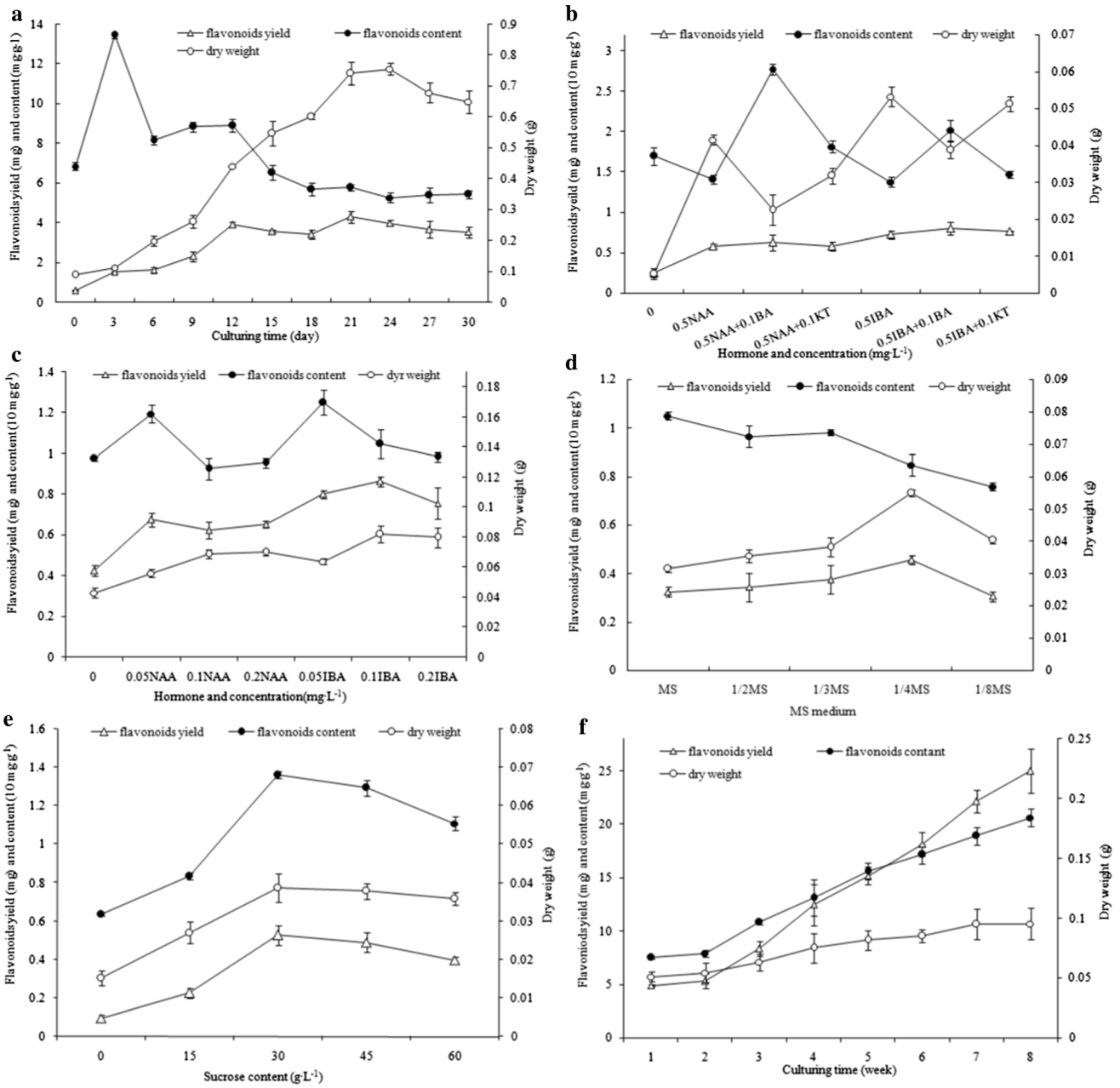


Fig. 1 Dry weight, flavonoid content and flavonoid yield curves of suspension culture cellus (**a**) and adventitious root (**b**, **c**, **d**, **f**) of *G. uralensis*. **a** Dry weight, flavonoid content and flavonoid yield curves of suspension culture cellus. **b**, **c** Effects of hormones impact on dry weight, flavonoid content and flavonoids yield of adventitious root which induced from callus (**b**) and from stem (**c**). **d–f** Effects of salt concentration (**d**), sucrose concentration (**e**) and culturing period (**f**) impact on the dry weight, flavonoid content and flavonoids yield of addvetitious roots which induced from stem

treatments, callus formed a white dot protuberance in the first 5 days of induction; adventitious roots formed from the 7th to 10th days.

The addition of auxin was conducive to the formation of adventitious roots, whether used alone or used in conjunction with the cytokinin, inducing callus to form adventitious roots, with a significant difference from the control. IBA is effective to induce adventitious root while used alone (Fig. 1b). The dry weight of the adventitious roots was 0.054 g in the medium with 0.5 mg L^{-1} IBA, and was significantly different from that of other groups. Adventitious root induction rates of IBA used alone or with 6-BA or KT were higher than those under the same level of NAA. The addition of cytokinin suppressed the formation of adventitious roots. The flavonoid content of adventitious roots in the medium with NAA 0.5 mg L^{-1} + 6-BA

0.1 mg L^{-1} was the highest, i.e., 27.79 mg g^{-1}, then was the combination of IBA 0.5 mg L^{-1} and 6-BA 0.1 mg L^{-1} with a flavonoid content of 20.293 mg g^{-1}, but the amount of adventitious roots was small, and the epidermis was easily broken. The flavonoid yield of IBA 0.5 mg L^{-1}, IBA 0.5 mg L^{-1} + 6-BA 0.1 mg L^{-1} and IBA 0.5 mg L^{-1} + KT 0.1 mg L^{-1} treatment groups was significantly higher than that of the NAA treatment groups, i.e., 0.733 g, 0.802 g and 0.754 g, respectively (Fig. 1b). The adventitious roots in the medium with 6-BA and KT were short, grew slowly and were not conducive to secondary culture. In general, IBA was the most suitable auxin for *G. uralensis* callus to induce adventitious roots.

Effect of different concentrations of NAA and IBA on Glycyrrhiza uralensis root tips

For inducing *G. uralensis* root, using NAA plus IBA or NAA alone was better than that of using IBA alone. In suspension culture medium with different concentrations of NAA and IBA, adventitious roots began to expand 3 days after induction. The epidermis of adventitious roots cultured in the medium containing NAA broke and gradually formed. With the NAA and IBA concentration increasing, adventitious roots began to form, and the broken epidermis dissolved into the medium, such that the culture solution was yellow and turbid.

The growth of adventitious roots was different in the media with IBA and NAA. The adventitious roots in the medium with IBA grew longer with many lateral roots, while those in the medium with NAA were stubby (Fig. 1c). The results showed that the adventitious root dry weight increased with increasing concentration of IBA and NAA. The dry weight of adventitious roots was the highest (0.0824 g) in the medium with IBA 0.1 mg L^{-1}, but when the concentration was higher than 0.1 mg L^{-1}, in either IBA or NAA treatment, the growth of adventitious root was suppressed (Fig. 1c).

The effect of auxin concentration on flavonoid synthesis in *G. uralensis* adventitious roots was also highly significant. The flavonoid content in adventitious roots decreased with increasing IBA and NAA concentrations in the medium. The flavonoid content (12.5484 mg g^{-1}) was highest in the medium with IBA 0.05 mg L^{-1} and differed significantly from other treatments with the IBA concentration ranging from 0.05 to 0.2 mg L^{-1}, indicating that a low concentration of IBA was conducive to the synthesis of flavonoids in adventitious roots (Fig. 1c). The yield of flavonoids of adventitious roots (0.8641 mg) was highest in the medium with IBA 0.1 mg L^{-1} and was significantly higher than that in other treatments (Fig. 1c). Therefore, 1/4-strength MS medium with IBA 0.1 mg L^{-1} is suitable for *G. uralensis* adventitious root culture.

Compared with control root tips that were brown, slender and single (Fig. 2a), roots induced by NAA were mostly single per block of callus, and their tips were yellow, shorter and intumescent (Fig. 2b). In response to NAA 0.5 mg L^{-1} + 6-BA 0.1 mg L^{-1}, adventitious roots were brown and short, and the epidermis was almost entirely broken (Fig. 2c). The adventitious roots under NAA 0.5 mg L^{-1} and KT 0.1 mg L^{-1} were yellow, most of which were in segments (Fig. 2d), whereas the adventitious roots induced by IBA 0.25 mg L^{-1} were yellow-white, numerous and slender, about 1–2 cm; grew rapidly; and formed as a single root or stellate-shaped (Fig. 2e, h). The adventitious roots in medium with IBA 0.5 mg L^{-1} plus 6-BA 0.1 mg L^{-1} or IBA 0.5 mg L^{-1} plus KT 0.1 mg L^{-1} were short and thick (Fig. 2f, g). The adventitious roots induced by IBA 1.0 mg L^{-1} were yellow-white, short and stellate-shaped (Fig. 2i), and the adventitious roots induced by IBA 2.0 mg L^{-1} were yellow and stellate-shaped, with a broken epidermis (Fig. 2j). The adventitious roots induced by IBA 4.0 mg L^{-1} were yellow, with root tip protuberance (Fig. 2k). Some adventitious roots in IBA 0.5 mg L^{-1} formed lateral roots (Fig. 2l).

The auxins commonly used in *G. uralensis* rooting are NAA and IBA. NAA is better in inducing roots than IBA. In different concentrations of NAA and IBA suspension culture medium, adventitious roots began to expand from the 3rd day. The epidermis of adventitious roots cultured in the medium containing NAA broke and gradually formed callus. As the concentration increased, the adventitious roots began to form, and the broken epidermis dissolved into the medium, such that the culture solution became yellow and turbid.

Adventitious root suspension culture

The effect of inorganic salt concentration on adventitious root growth

The results showed that the concentration of inorganic salts in the medium had a strong impact on adventitious root growth and the accumulation of flavonoids. 1/4-strength MS medium was the most conducive to the growth of adventitious roots; a very low (1/8 MS) or high (MS) concentration of inorganic salt could inhibit adventitious root growth. The roots were white initially; lateral roots formed from the 2nd and 3rd day; apicals began to elongate 3–5 days later; 10 days later the roots gradually became yellow-white and intertwined with each other; and the roots were yellow-brown 3 weeks later. Adventitious root apicals elongated slowly; the number of lateral roots was lower and the lengths of lateral roots was shorter in MS medium. With a decrease in the major element in the medium and the number of lateral roots, the length of

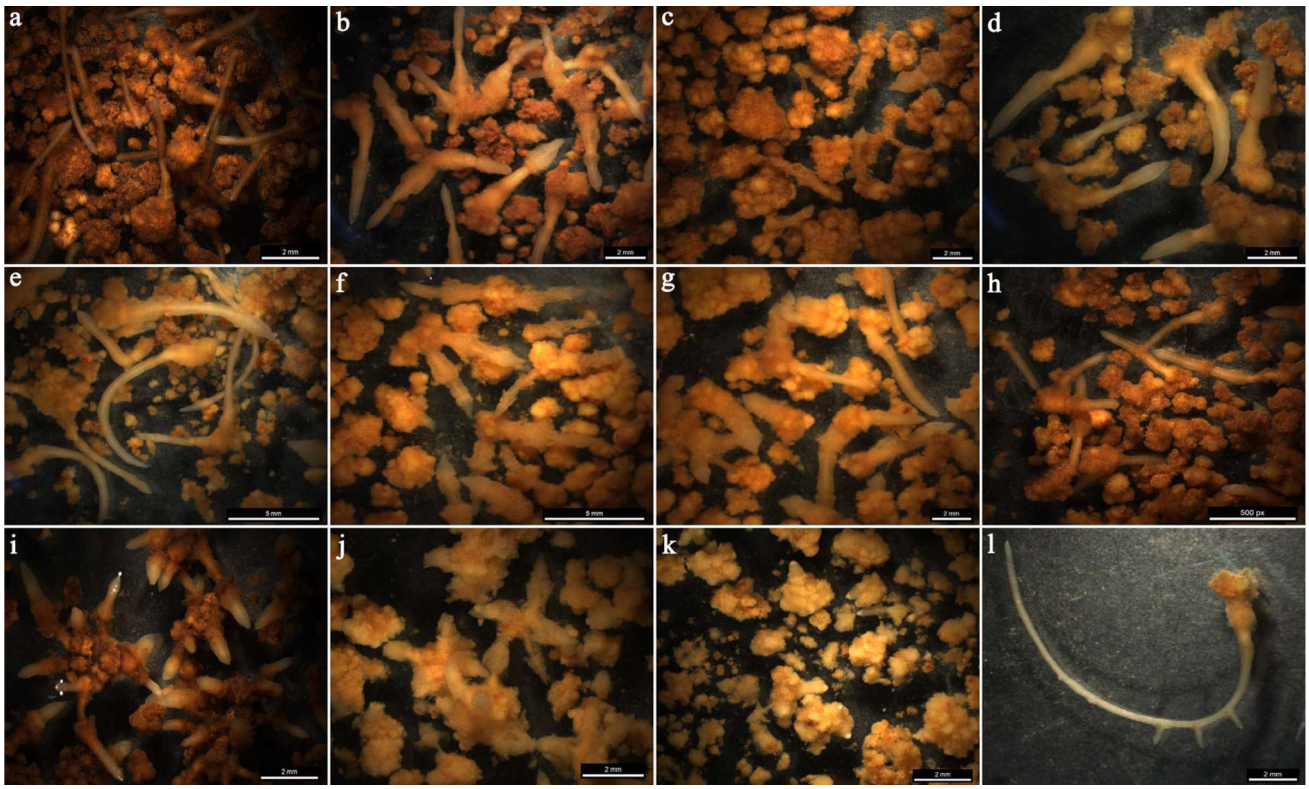


Fig. 2 Adventitious roots and callus in different media. **a** Control (× 10): brown, slender, single; **b** NAA0.5 mg L^{-1} (× 12.5): yellow, root tip intumescent, shorter; **c** NAA0.5 mg L^{-1} + 6-BA0.1 mg L^{-1} (× 12.5): brown, short, epidermis broken; **d** NAA0.5 mg L^{-1} + KT0.1 mg L^{-1} (× 10): yellow, in segments; **e** IBA0.5 mg L^{-1} (× 7.3): yellow-white, slender; **f** IBA0.5 mg L^{-1} + 6-BA0.1 mg L^{-1} (× 7.3): yellow-brown, short and thick, epidermis broken; **g** IBA0.5 mg L^{-1} + KT0.1 mg L^{-1} (× 12.5): yellow, short and thick; **h** IBA0.25 mg L^{-1} (× 7.3): brown, slender, single; **i** IBA1.0 mg L^{-1} (× 10): yellow-white, short, stellate-shaped; **j** IBA2.0 mg L^{-1} (× 10): yellow, stellate-shaped, epidermis broken; **k** IBA4.0 mg L^{-1} (× 10): yellow, root tip protuberance; **l** IBA0.5 mg L^{-1} (× 10): Adventitious root with lateral roots (color figure online)

lateral roots was significantly longer than that in the MS medium. The adventitious root apicals in the 1/4-strength MS medium grew obviously strong. The difference in fresh weight, dry weight and the length reached a significant level compared with other treatments (Table 1, Fig. 1d). The number of lateral roots was significantly increased, and fresh weight and dry weight increased rapidly. Taproots were strong. Therefore, the 1/4-strength MS medium was more suitable for the growth of adventitious roots.

With the decreasing concentration of inorganic salts in the medium, the content of flavonoids in adventitious roots showed a decreasing trend. The flavonoid content was the highest (10.4317 mg g^{-1}) in MS medium. The yield of flavonoids in adventitious roots was highest, i.e., 0.4638 mg g^{-1}, in 1/4-strength MS medium.

Sucrose concentration Roots were hardly grown without sucrose in medium. Whereas a lower sucrose concentration of 15 g L^{-1} was added, and fewer yellowish-white roots were growing and the dry matter was 9.48%. With increasing sucrose concentration up to 30 g L^{-1}, both fresh and dry weights of roots reached the highest, 0.3759 g and 0.0387 g, respectively, and reached a significant level compared with other sucrose concentration treatments. With increasing sucrose concentration, the nutrient solution gradually turned deep yellowish-brown, and the fresh weight decreased gradually, which showed that excessive sucrose concentration restrained root growth. Similar to the root growth trend, the effect of sucrose concentration on flavonoid synthesis increased first and then decreased. When the sucrose concentration was 30 g L^{-1}, the content and production reached the highest values of 13.6215 mg g^{-1} and 0.5267 mg g^{-1}, respectively, which means that a sucrose concentration of 30 g L^{-1} is suitable for *G. uralensis* growth (Fig. 1e).

Period culture The roots grew slowly in vitro 1–2 weeks after inoculation (Fig. 1f), but grew rapidly during the 3rd–7th weeks, and the increase in roots remained at a stable level during the 7th–8th weeks. Whereas the flavonoid yield exhibited different trends from those of root growth during the 1–8-week period, both the *G. uralensis* flavonoid content and flavonoid yield increase diminished with prolonged culture time. In conclusion, the 7th day was

Table 1 Effects of salt in medium on the growth of Licorice adventitious roots

Medium	The length of taproot (mm)	The number of lateral roots	The length of lateral root (mm)	Fresh weight (g)	Dry weight (g)
MS	36.24d	6.406b	2.118d	0.322 ± 0.01b	0.0314 ± 0.001c
1/2MS	56.13ab	6.958b	7.017ab	0.338 ± 0.02b	0.0356 ± 0.002bc
1/3MS	51.92b	7.125b	7.934b	0.376 ± 0.03b	0.0382 ± 0.003bc
1/4MS	58.87a	10.958a	8.608a	0.472 ± 0.01a	0.055 ± 0.001a
1/8MS	42.84c	6.417b	4.316c	0.368 ± 0.02b	0.0402 ± 0.001b

Duncan multiple comparison, the same letter means the difference is not significant ($P < 0.05$)

Table 2 The flavonoids yield in callus and adventitious roots

Cultures	Culturing time (days)	Mediums (mg L^{-1})	Flavonoid content (mg g^{-1})	Flavonoid yield (mg g^{-1})	Production efficiency (mg g^{-1} day^{-1})
Suspension callus	21	MS + 2,4-D0.2 + NAA0.5 + 6-BA 0.5	5.783 ± 0.142b	4.286 ± 0.315b	0.204
Adventitious roots inducing in stem	49	1/4MS + IBA 0.1	19.354 ± 0.975a	0.927 ± 0.054c	0.095
Adventitious roots inducing in callus	30	1/2MS + IBA 1.0	19.918 ± 0.109a	8.255 ± 0.214a	0.550

Means followed by the same letter with in each column are not significantly different at the $P < 0.05$ indicated by a Duncan s multiple range test. Culture for 30 days

the optimum period for harvesting *G. uralensis* roots in vitro.

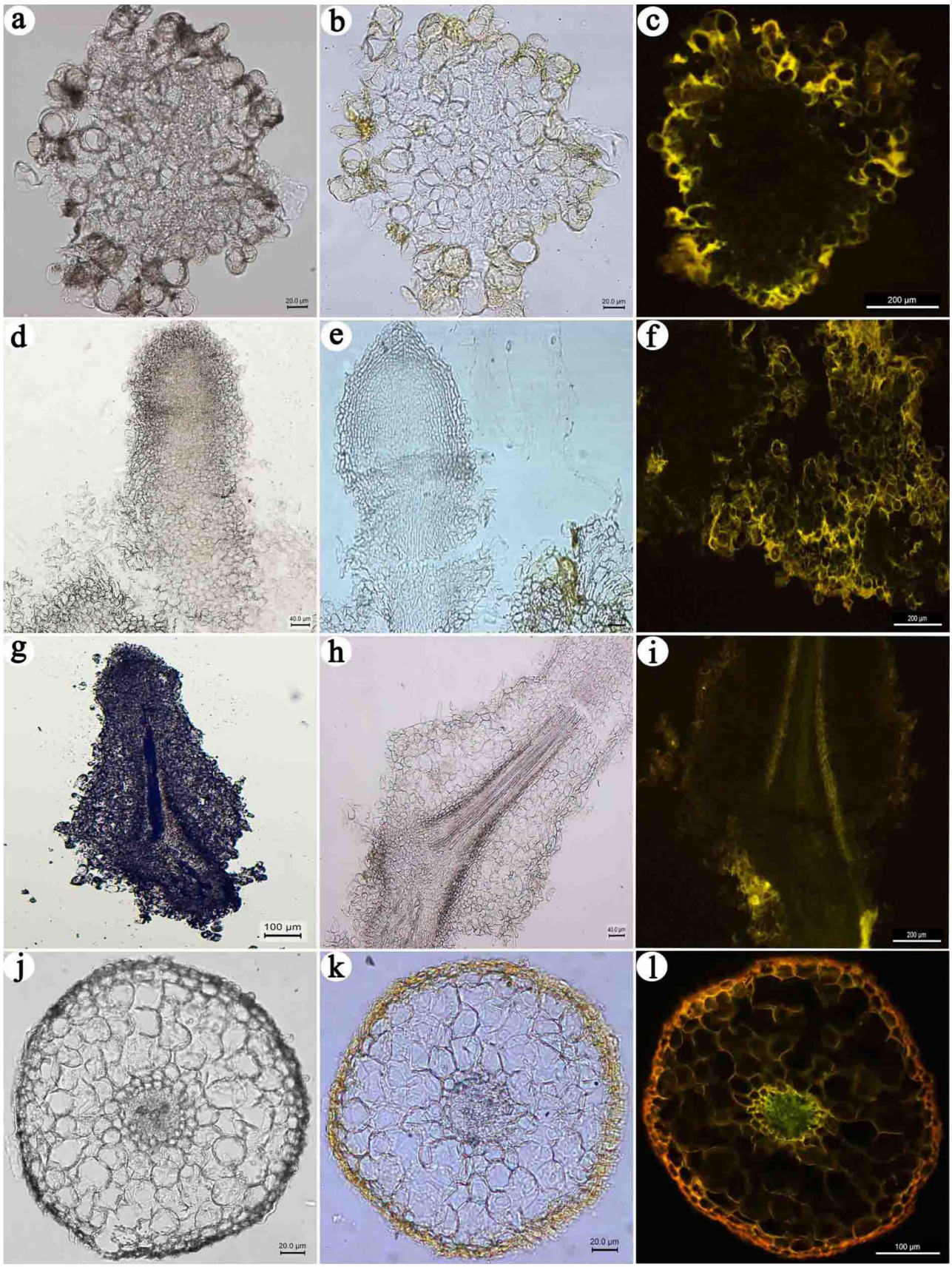
Comparison of the content and yield of flavonoids in different cultures

The experimental results proved that the best harvest time of the suspension callus was the 21st day, and that of the suspension apical was the 49th day. In this study, we compared the content and yield of *G. uralensis* flavonoids under different cultures. The flavonoid content in apicals was 19.354 mg g^{-1}, which was higher than that in suspension callus, but the growth of roots was so slow that the flavonoid yield was significantly lower than that in suspension callus. While the adventitious roots were induced from suspension callus about 30 days later, the flavonoid content was significantly higher, 19.918 mg g^{-1} (Table 2), about 4-fold that of the initial suspension callus. Therefore, the generation of adventitious roots promoted the accumulation of flavonoids.

The flavonoid contents of apicals cultured in vitro were similar to the flavonoid contents of adventitious roots induced from the suspension callus, but because of the slow growth of roots, the flavonoid yield of apicals cultured in vitro was the lowest. Therefore, the expanding culture of apicals cannot accumulate numerous flavonoids. In the current experimental results, the accumulation of flavonoids in different cultures in descending order was adventitious roots, suspended callus, root and tips cultured in vitro.

The flavonoid content did not gradually increase with the extension of the incubation time during the first week, probably because the callus cells synthesized flavonoids in a suitable growth environment. If there are no external stimuli, then fewer flavonoids are synthesized. Many medicinal plants accumulate secondary metabolites from apical meristem culture gradual accumulation of flavonoids, although the roots grow more slowly, but the final flavonoid yield obtained from culture alone or adventitious root tips due to the higher flavonoid content root itself. In the adventitious callus induction process, flavonoids gradually increased, the same as the gradual accumulation of

Fig. 3 Accumulation of flavonoids during adventitious root formation process. **a** callus-unstained (× 200); **b** callus-NaOH staining (× 200); **c** callus-$AlCl_3$ ethanol staining (× 100); **d** the 10th day of adventitious root induction-unstained (× 200); **e** the 10th day of adventitious root induction-NaOH staining (× 200); **f** the 10th day of adventitious root induction-$AlCl_3$ ethanol staining (× 100); **g** the 20th day of adventitious root induction-unstained (× 40); **h** the 20th day of adventitious root induction-NaOH staining a less (× 200); **i** the 20th day of adventitious root induction-$AlCl_3$ ethanol staining (× 100); **j** the 30th day of adventitious root induction-unstained (× 100); **k** the 30th day of adventitious root induction-NaOH staining (× 100); **l** the 30th day of adventitious root induction-$AlCl_3$ ethanol staining (× 100)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adventitious roots, so adventitious co-culture with callus material accumulated the maximum amount of flavonoids.

In Wang's (2013) study, the contents in different *G. uralensis* materials were analyzed using cluster analysis. They concluded that adventitious roots had a greater capability of flavonoid production compared to seedlings, callus and cells, but another study showed that the number of flavanones was lower in cultured *Glycyrhiza* cells (6.31 mg g^{-1}) than in native cells (9.82 mg g^{-1}) (Man et al. 2013). In our study, the content of flavonoid in callus was lower than that in native cells, and adventitious roots showed a fairly high capacity to produce flavonoids. Li et al. (2012) studied the efficient genetic transformation of licorice (*Glycyrrhiza inflata* Batalin) cells in suspension culture using Agrobacterium tumefaciens-mediated T-DNA delivery. The results showed that the introduced genes had no discernable effect on cell growth or the accumulation of total licorice flavonoids in the transgenic cell lines. It seems that the technique of molecular biology is also likely to be a method to improve flavonoid synthesis, but not the most effective one. We conclude that callus induction of adventitious roots was the most suitable method for obtaining flavonoids effectively.

Flavonoid histochemical localization

NaOH can result in a color reaction with flavanone compounds to make chalcone, showing a yellow color. The suspended secondary culture callus epidermal cells were yellow, but the internal region was not stained (Fig. 3b). In the early days of rooting (10 days), only the callus epidermis was yellow, with less staining of the adventitious roots (Fig. 3e), suggesting low flavonoid accumulation in the early induction of adventitious roots. On the 20th day, the adventitious root endodermis was light yellow, and the color of the epidermis was not obvious (Fig. 3h), whereas the callus was stained deeper than the epidermis of adventitious root. On the 30th day, adventitious root epidermal cells were yellow, but other cells were not stained (Fig. 3k).

The $AlCl_3$ ethanol solution can react with flavonoids to produce a yellow complex, which presented blue-white, yellow, or yellow-green fluorescence under a fluorescence microscope. Results showed that suspended callus parenchyma cells presented clear yellow-green and were mainly distributed in the cell wall and intercellular space. In the early days of rooting (10 days), both callus (Fig. 3c) and adventitious roots (Fig. 3f) presented yellow-green fluorescence, obviously brighter than the similar tissues or organs stained by NaOH. On the 20th day, adventitious root endodermal cells presented yellow-green fluorescence; epidermal cells also had considerable light green fluorescence (Fig. 3i). On the 30th day, adventitious root epidermal cells presented orange-yellow fluorescence; the epidermal cells presented yellow-green fluorescence; xylem parenchyma cells also had a small amount of blue-green fluorescence (Fig. 3l). The results were consistent with the results of NaOH histochemical localization, but the tissues or organs stained by $AlCl_3$ ethanol solution is more obvious than NaOH staining. Therefore, $AlCl_3$ ethanol solution was better than NaOH aqueous solution in terms of chromogenic and localization effects. During the primary growth of adventitious roots, flavonoids may first be transported from callus epidermal cells to the endodermis of adventitious roots and eventually accumulate in the epidermal cells.

The cycle of suspension callus culture was 18–21 days. IBA was more conducive than NAA in inducing *G. uralensis* adventitious roots, but the addition of 6-BA and KT suppressed the formation of adventitious roots. The flavonoid content and yield reached the highest values of 19.962 mg g^{-1} and 1.232 mg g^{-1}, respectively, when the IBA concentration was 1.0 mg L^{-1}. The study of histochemical localization of flavonoids suggested that *G. uralensis* flavonoids were mainly distributed in the epidermal cells of the callus outer layers. There was no accumulation of flavonoids in the roots in the early stage of the induction of adventitious roots. With the primary growth of adventitious roots, flavonoids gradually distributed in the cell wall and cell gaps of the epidermis and endodermis of adventitious roots. The best medium for the induction and growth of adventitious roots was 1/4-strength MS + 0.1 mg L^{-1} IBA. In this medium, apicals grew faster, and the yield of flavonoids was the highest. The content of flavonoids in adventitious roots and apicals cultured in vitro was higher than that in suspension callus and reached 3.87 times the callus flavonoid content.

Conclusion

We concluded that calli inducing adventitious roots and apicals for 30 days obtained the highest yield of flavonoid. The best medium for induction and growth of adventitious roots was 1/4-strength MS + 0.1 mg L^{-1} IBA. In this medium, apicals grew faster, and the yield of flavonoids was the highest. $AlCl_3$ ethanol solution was better than NaOH aqueous solution in terms of the chromogenic and localization effects on flavonoid. Using in vitro culture instead of wild culture is an effective method for producing flavonoids.

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Author contributions SG and YZ designed the study and conceived the experiments. YZ and YJ carried out the experiments, and analyzed data, drafted the manuscript. YS partly participated writing manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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