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ORIGINAL PAPER

Effects of nitrogen source and phosphate concentration on biomass and metabolites accumulation in adventitious root culture of *Glycyrrhiza uralensis* Fisch

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Abstract We investigated the influence of ammonia/nitrate ratio and phosphate concentration on biomass and accumulation of metabolites in adventitious roots of *Glycyrrhiza uralensis* Fisch. A NH_4^+/NO_3^- ratio of 10:20 was optimal for the production of biomass (0.30 g dry weight) as well as polysaccharide (18.98 mg g^{-1}) and glycyrrhetinic acid (0.31 mg g^{-1}). The content of glycyrrhizic acid (0.47 mg g^{-1}) and flavonoid (8.11 mg g^{-1}) reached the optimum at an ammonia/nitrate ratio of 15:15 and 20:10, respectively. In case of phosphate concentration, a higher growth rate (9.18) and content of polysaccharide (15.66 mg g^{-1}) was obtained at 1.25 mM phosphate concentration. However, 0.625 mM phosphate was favorable for the content of flavonoid (7.54 mg g^{-1}) and glycyrrhizic acid (0.57 mg g^{-1}). The content of glycyrrhetinic acid (0.32 mg g^{-1}) reached the peak when treated with 0.3125 mM phosphate. A scale-up culture of adventitious roots was established using a balloon-type bubble bioreactor (BTBB). Maximum growth rates of 25.95- and 22.44-fold were obtained in 3 and 5 L BTBBs, respectively, which was higher than that in 0.5 L shake flask (18.36). The contents of flavonoid (7.60 mg g^{-1}) and polysaccharide (15.09 mg g^{-1}) reached the peak in 5 L and 3 L BTBB, respectively. The contents of glycyrrhizic and glycyrrhetinic acid were a little higher than that in BTBBs.

Keywords Adventitious root · *Glycyrrhiza uralensis* Fisch · Nitrogen source · Phosphate concentration · BTBB

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Introduction

Glycyrrhiza uralensis Fisch has been widely used for over 3,000 years as a traditional oriental herb (Zhang et al. 2009). In China, it was used in a large number of traditional Chinese medicinal prescriptions for the treatment of cancer, hepatitis as well as detoxication (Li et al. 2011). Glycyrrhizic and glycyrrhetinic acid, as main saponins, are the most important pharmacologically active component in *G.uralensis*, exhibiting anti-inflammation, anti-virus and anti-HIV properties (Wang et al. 2012). Flavonoids, secondary metabolites of the plant, are widely used for antioxidant (Man et al. 2013) and anti-tumor (Zhang et al. 2009). The polysaccharide, primary metabolites in *G. uralensis* has drawn the attention of researchers due to its physical and functional properties (Wan and Cheng 2009).

In recent years, *G. uralensis* has been increasingly used as a health additive formulated into kinds of commercial products such as food, health products, cosmetics (Man et al. 2013). Recently, natural sources of wild *G. uralensis* are very limited because of over-exploitation (Dong et al. 2012). The current supply of *G. uralensis* mainly depends on field culture, which is an extremely time-consuming and labor-intensive process. However, there is a great demand and scant supply for *G. uralensis*. As a result, cell, tissue, and organ culture has been exploited as an alternative for

more efficient and controllable production of *G. uralensis* and its active constituents.

Nitrogen source has been an essential factor that influences the quality of cell and root growth in a species-dependent manner (Gorret et al. 2004). The significant role of the nitrogen source has been well demonstrated in many cultures such as *Panax ginseng* (Kim et al. 2005), *Echinacea angustifolia* (Wu et al. 2006), *Eleutherococcus koreanum* Nakai (Lee and Paek 2012) and so on. Phosphate source is another important element that constitutes nucleotide, phosphatides and adenosine triphosphate and so on (Wang et al. 2009; Zhong and Zhu 1995). Moreover, it has an important influence on the growth and active components synthesis which has been proved by many reports (Liu and Zhong 1998; Jiang et al. 2006; Huang et al. 2010).

Callus induction and suspension cell culture systems as well as hairy root culture have been studied to obtain flavonoids in *G. uralensis* (Guo et al. 2012; Yang et al. 2008; Zhang et al. 2009). Wang et al. (2012) made content comparison with regards to seeding, callus, cell and adventitious root in *G. uralensis*. However, to our knowledge, there have been few reports on adventitious root culture of *G. uralensis* with regard to optimization of culture conditions. The objective of this study was to optimize the ratio of ammonium to nitrate and phosphate concentration for the large-scale productivity of biomass and bioactive compounds in adventitious roots of *G. uralensis*.

Materials and methods

Plant material

Seeds of *G. uralensis* were supplied by Beijing materia herbal medicine technology Co. (Beijing, China).

Seeds' surface were washed under running tap water for 2 h and then they were further sterilized with 75 % (v/v) ethanol for 30 s, immersed in 2 % (v/v) NaClO solution for 30 min and rinsed with sterile distilled water. The sterilized seeds were inoculated into conical flasks containing 50 mL Murashige and Skoog (MS) medium supplemented with 30 g L^{-1}sucrose and 6.5 g L^{-1} agar. Cultures were maintained at 23 ± 2 °C with 54–72 μmol m^{-2} s^{-1} light intensity under a 16/8 h (day/night) photoperiod. After 4 weeks, they grew into plantlets and the root explants were cut into 1 cm length for the subsequent experiments.

Induction of adventitious roots and their maintenance

Adventitious roots of *G. uralensis* were induced from root explants on half-strength MS medium supplemented with 30 g L^{-1} sucrose, 1 mg L^{-1} IBA and 6.5 g L^{-1} agar. Those induced adventitious roots were inoculated into 250 mL Erlenmeyer flasks containing 100 mL half-strength MS medium supplemented with 1 mg L^{-1} IBA and 30 g L^{-1}sucrose. Cultures were maintained at 23 ± 2 °C in the dark on gyratory shakers at 120 rpm and were sub-cultured every 30 days.

Effects of nitrogen source and phosphate concentration on adventitious root growth and metabolite accumulation

Adventitious roots (6 g L^{-1}) were inoculated into 1/2 MS medium supplemented with 30 g L^{-1} sucrose, 1 mg L^{-1} IBA and various ratios of NH_4^+/NO_3^- (0/30, 10/20, 15/15, 20/10, 30/0, using NH_4Cl and KNO_3) at the constant nitrogen source level of 30 mM, which was basically the level of that in a half-strength MS medium. Adventitious roots (6 g L^{-1}) were inoculated into 1/2 MS medium supplemented with 0, 0.3125, 0.625, 1.25, 1.875 mM phosphate. The ratio of NH_4^+/NO_3^- was 10:20 and the culture conditions were the same as described above. Each treatment was repeated three times.

Scale-up culture of adventitious root from shake flask to bioreactor

Adventitious roots were further proliferated in 0.5 L Erlenmeyer flasks, 3 and 5 L balloon-type bubble bioreactors (BTBBs) with 0.2, 2 and 3 L working volume, respectively. The half-strength MS medium was supplemented with 1 mg L^{-1} IBA and 30 g L^{-1} sucrose. Root inoculum was adjusted to a density of 1.0 g L^{-1} and the air volume was adjusted to constant flow rate of 0.4 air volume/culture volume/min (vvm) during BTBBs culture according to previous experimental optimization. Cultures were maintained at 23 ± 2 °C in the dark. Each treatment was repeated three times.

Determination of root biomass

After 30 days culture, the harvested roots were washed with running water twice, and then rinsed with distilled water three times. Fresh weight (FW) was determined after blotting the washed roots on filter paper. The fresh roots were then dried in vacuum at 50 °C for 2 days to constant dry weight (DW). The growth rate was calculated as (harvested dry weight−inoculated dry weight)/inoculated dry weight.

Determination of total flavonoid and polysaccharides

Samples of adventitious roots were ground to a fine powder and extracted twice with 30 mL of 75 % ethanol for 30 min at 60 °C in an ultrasonic bath (Kunshan, China). After filtration, the extract was evaporated to dryness, and

then dissolved in 2 mL 75 % ethanol. The content of flavonoid was determined as described in the paper (Man et al. 2013) with Liquiritin as a reference standard ($A = 0.0065C - 0.0046$, $r = 0.9976$).

In terms of the extraction of polysaccharides, the residue after filtration was drying and extracted three times with 25 mL distilled water for 1 h at 100 °C. The extract was diluted to 50 mL for quantification. The content of polysaccharide was analyzed using the method of sulphuric acid—anthrone reported by Chen et al. (2005) with glucose as a reference standard ($A = 0.0073C + 0.0213$, $r = 0.9962$).

Determination of the content of glycyrrhizic and glycyrrhetinic acid

Extracts of ethanol were analyzed by HPLC (Agilent1100, PaloAlto, CA) using a promosil C18 column (4.6 mm × 250 mm, 5 μm; Agela, Tianjin, China) to determine the content of glycyrrhizic and glycyrrhetinic acid. Mobile phase was composed of A-formic acid (0.04 %, v/v) and B-acetonitrile. Gradient elution profile (A:B) was 0–4 min, 80:20; 20 min, 62:38; 25 min, 45:55; 38–40 min, 10:90. The detection wavelength was set at 250 nm. The flow rate was 1.0 mL min^{-1} and the column temperature was maintained at 30 °C.

Statistical analysis

The statistical analysis was performed according to the V 17.0 SPSS system. Mean and standard errors were used throughout, and the statistical significance between the mean values was assessed applying a Duncan's multiple range tests. A probability of $P < 0.05$ was considered significant.

Results and discussion

Effects of nitrogen source on biomass production and metabolites accumulation

Nitrogen source significantly influenced the biomass accumulation of *G. uralensis* adventitious roots. The optimum biomass of 5.08 g $flask^{-1}$ FW and 0.30 g $flask^{-1}$ DW were obtained when the NH_4^+/NO_3^- ratio was 10:20 (Table 1). The highest polysaccharide content (18.98 mg g^{-1}) was also obtained under a NH_4^+/NO_3^- ratio of 10:20; whereas, the content of total flavonoid reached the peak at a NH_4^+/NO_3^- ratio of 20:10. In case of saponin, glycyrrhizic acid was optimum when the NH_4^+/NO_3^- ratio was 15:15 and the content of glycyrrhetinic acid reached the peak at a NH_4^+/NO_3^- ratio of 10:20. These results suggest that the NH_4^+/NO_3^- ratio of 10:20 was favorable to generate the optimum biomass and accumulation of polysaccharide and glycyrrhetinic acid. It was a general trend that a lower NH_4^+/NO_3^- ratio is more beneficial for plant cell growth (Panda et al. 1992; Kaul and Hoffman 1993; Yu et al. 2001), and our study also supported this point (Table 1). It may be because ammonium diffuses easily and accumulates into the cell which becomes toxic if not immediately metabolized, so the ammonium must control to a low concentration (Zhang et al. 2011).

With ammonium as the sole N source (i.e. $NH_4^+/NO_3^- = 30/0$), the root scarcely grew in the culture (Table 1). Zhong and Wang (1998) demonstrated that high ammonium concentration had an inhibitory effect on cell growth in culture of *Panax quinquefolium*. Kaul and Hoffman (1993) also reported that ammonium as the sole N source inhibited callus growth of *Pinus strobus*. We can suppose that the sole nitrogen source of ammonia was unfavorable for root growth. In the case of nitrate as the sole N source, the root grew a little better than that with totally ammonium in the medium. In the other case where ammonium and nitrate were both supplied into the medium, the root grew well compared with nitrate or ammonium as the sole N source in the medium. The existence of ammonium could suppress nitrate assimilation which would result in medium acidification. Generally speaking, the plant cell utilized the ammonium priority in the medium. However, excessive ammonium has toxic effects on plant cell and root growth (Wang et al. 2009). Although nitrate is safe for plant, over addition may result in over-acidification of the medium which is unfavorable to root growth (Zhang et al. 2011).

Table 1 Effects of ammonia/nitrate ratio on biomass production and metabolites accumulation in adventitious root culture of *G. uralensis*

NH_4^+/NO_3^-	Fresh weight (g/flask)	Dry weight (g/flask)	Growth ratio	Glycyrrhizic content (mg/g)	Glycyrrhetinic content (mg/g)	Flavonoid content (mg/g)	Polysaccharide content (mg/g)
0:30	2.08 ± 0.15d	0.14 ± 0.01d	3.10 ± 0.15d	0.43 ± 0.03c	0.21 ± 0.03d	5.19 ± 0.42e	8.75 ± 0.58d
10:20	5.08 ± 0.36a	0.30 ± 0.02a	8.12 ± 0.31a	0.39 ± 0.02e	0.31 ± 0.02a	7.45 ± 0.31c	18.98 ± 0.67a
15:15	4.31 ± 0.13c	0.26 ± 0.05c	6.65 ± 0.15c	0.47 ± 0.02a	0.24 ± 0.02c	8.02 ± 0.29b	7.11 ± 0.14e
20:10	4.64 ± 0.19b	0.26 ± 0.03bc	6.78 ± 0.19bc	0.41 ± 0.04d	0.26 ± 0.04bc	8.11 ± 0.34ab	9.59 ± 0.53c
30:0	0.82 ± 0.08e	0.06 ± 0.005e	0.67 ± 0.05e	0.45 ± 0.06b	0.18 ± 0.06e	5.53 ± 0.17d	12.91 ± 0.47b

The data were collected after 30 days of culture in a 250 mL Erlenmeyer flask containing 100 mL medium. Mean ± standard error of three replicates; mean followed by different letters within a column is significantly different at $P < 0.05$ according to Duncan's multiple range test

Nitrogen source significantly affects the plant cell and tissue growth and metabolite formation (Cui et al. 2010b). Ammonium and nitrate as nitrogen source have different effects on cell and tissue growth and active component production (Zhang et al. 1996). It was apparent that nitrate was more necessary than ammonium for root growth and metabolites accumulation. Similar observations were also reported by Nagella and Murthy (2011) who concluded that biomass and withanolide-A were larger, when the NO_3^- was higher than that of NH_4^+ concentration in cell suspension cultures of *Withania somnifera* (L.) Dunal. In adventitious shoot cultures of *Bacopa monnieri* (L.), the number of adventitious shoot biomass and bacoside A content were optimum at a lower NH_4^+/NO_3^- ratio (Naik et al. 2011). Pan et al. (2004) indicated that cell dry weight was improved under lower NH_4^+/NO_3^- ratio in cell suspension cultures of *Camptotheca acuminate*. Our results were similar to the literatures. Therefore, we can draw a conclusion that most of the plants need a lower NH_4^+/NO_3^- ratio.

Effects of phosphate concentration on biomass production and metabolites accumulation

The biomass accumulation of *G. uralensis* adventitious roots was highly affected by phosphate concentration. In the range of phosphate level from 0 mM to 1.875 mM, the maximum root dry weight (0.34 g·flask) and the highest growth rate (9.18) were observed in the culture treated with 1.25 mM phosphate (Table 2). When phosphate concentration exceeded 1.25 mM, the biomass of adventitious roots showed a drop in growth rate (7.52). We can infer that higher phosphate over 1.25 mM phosphate concentration was detrimental to the root growth. However, excessively lower phosphate was also harmful to adventitious root growth which cannot absorb enough phosphate to metabolite. According to Table 2, we can see that it was unfavorable to adventitious root proliferation in the phosphate-free medium (i.e., 0 mM phosphate) whose growth ratio was only 2.49. We can conclude that adventitious root growth was unfavorable in the medium without phosphate source. The similar phenomenon was also reported in rice cells (Wen and Zhong 1997). In terms of *Catharanthus roseus* (L.), Sakano et al. (1995) reported that cells could not proliferate at all in medium lacking phosphate source. Phosphate source plays an important role in the production of biomass and accumulation of metabolites. It also participates in various kinds of energy metabolism and material biosynthesis (Huang et al. 2010).

The results exhibited in the culture treated with 1.25 mM phosphate, the content of polysaccharide reached the maximum i.e. 15.66 mg g^{-1}. The highest amount of flavonoid (7.54 mg g^{-1}) and glycyrrhizic acid (0.57 mg g^{-1}) was accumulated with 0.625 mM phosphate. However, 0.3125 mM phosphate was optimum for the accumulation of glycyrrhetinic acid (0.32 mg g^{-1}). We can see that the accumulation of polysaccharide required higher phosphate (1.25 mM), however the accumulation of flavonoid and saponin (glycyrrhizic and glycyrrhetinic acid) required lower phosphate (0.625 and 0.3125 mM). We can suppose that it may relate to the metabolic pathways of primary metabolites (polysaccharide) and secondary metabolites (flavonoid and saponin) (Table 3).

Phosphate is an essential nutrient which participates in metabolite formation and energy metabolism and biosynthesis. In adventitious root culture of *P. ginseng* CA (Huang et al. 2010), it was found that the root growth ratio reached its peak at the concentration of 0.625 mM phosphate, however, the maximum ginsenoside content was achieved at 1.25 mM. Zhong and Zhu (1995) concluded that the highest production and yield of ginsenosides were obtained at 1.25 mM of medium phosphate in suspension cell culture of *Panax notoginseng* which was somewhat similar with our results. This phenomenon showed that the effect of phosphate concentration on adventitious root was very complicated, which depended on both species and kinds of secondary metabolites.

Table 2 Effects of phosphate concentration on biomass production and metabolites accumulation in adventitious root culture of *G. uralensis*

Phosphate source	Fresh weight (g/flask)	Dry weight (g/flask)	Growth ratio	Glycyrrhizic content (mg/g)	Glycyrrhetinic content (mg/g)	Flavonoid content (mg/g)	Polysaccharide content (mg/g)
0	1.58 ± 0.09e	0.12 ± 0.02e	2.49 ± 0.16e	0.43 ± 0.03d	0.27 ± 0.03de	4.71 ± 0.21e	10.90 ± 0.46d
0.3125	5.41 ± 0.19bc	0.29 ± 0.05bc	7.66 ± 0.15bc	0.50 ± 0.03bc	0.32 ± 0.02a	6.61 ± 0.33 cd	11.03 ± 0.33 cd
0.625	4.15 ± 0.12d	0.26 ± 0.05d	6.75 ± 0.15d	0.57 ± 0.05a	0.27 ± 0.02cde	7.54 ± 0.42a	9.68 ± 0.24e
1.25	5.95 ± 0.26a	0.34 ± 0.06a	9.18 ± 0.27a	0.49 ± 0.04c	0.28 ± 0.04bcd	6.83 ± 0.24bcd	15.66 ± 0.41a
1.875	5.34 ± 0.56c	0.28 ± 0.02c	7.52 ± 0.31c	0.40 ± 0.02e	0.26 ± 0.03e	6.52 ± 0.26d	13.01 ± 0.32b

The data were collected after 30 days of culture in a 250 mL Erlenmeyer flask containing 100 mL medium. Mean ± standard error of three replicates; mean followed by different letters within a column is significantly different at $P < 0.05$ according to Duncan's multiple range test

Scale-up of adventitious root in *G. uralensis*

The growth of adventitious root in Erlenmeyer flasks and BTBB was shown in Fig. 1. Adventitious root growth differed significantly between Erlenmeyer flasks and bioreactor cultures. Roots began to differentiate after 7 d and 12 d in the liquid cultures of Erlenmeyer flask and bioreactor, respectively. The growth ratio of 25.95 and 22.44 was obtained in 3 L (53.9 g FW, 2.52 g DW) and 5 L BTBB (90.22 g FW, 4.22 g DW) which were significantly more than Erlenmeyer flasks cultures of 18.36 (9.68 g FW, 0.68 g FW). Gradually scale-up culture of adventitious roots increased the root biomass as well as the contents of polysaccharide and flavonoid. However, there was no significant change in the content of glycyrrhizic and glycyrrhetinic acid in bioreactor compared with the Erlenmeyer flask.

The biomass increase might be attributed to the culture conditions in bioreactor which can be optimized by real-time manipulation of temperature, pH and oxygen in the medium (Wang et al. 2012). We supposed that the culture condition may have an influence on the active component synthesis of *G.uralensis*. In adventitious root culture of *P. ginseng*, Choi et al. (2000) demonstrated that the content of total saponin in the pilot-scale culture was similar to that obtained in the small-scale bioreactor. Our result regarding the content of saponin was similar to the viewpoint.

The use of bioreactors has resulted in the development of a technology for large-scale biomass of adventitious roots culture (Cui et al. 2010a). Similar observation was also reported in *A. membranaceus* adventitious root culture (Wu et al. 2011). The maximum growth rate of *A. membranaceus* in 5 L bioreactors was tenfold after 40 days of culture higher than that cultivated in flasks. This is also

Table 3 The biomass and contents of active component in adventitious root of *G. uralensis* from 0.5 L shake flask to 5 L bioreactor

Culture methods	FW (g/vessel)	DW (g/vessel)	Growth ratio	Glycyrrhizic content (mg/g)	Glycyrrhetinic content (mg/g)	Flavonoid content (mg/g)	Polysaccharide content (mg/g)
0.5 L Shake flask	9.68 ± 0.23	0.68 ± 0.07	18.36 ± 0.21	0.49 ± 0.04	0.26 ± 0.02	5.03 ± 0.54	9.78 ± 0.23
3 L Bioreactor	53.90 ± 1.02	2.52 ± 0.19	25.95 ± 0.32	0.43 ± 0.02	0.25 ± 0.01	6.96 ± 0.29	15.09 ± 0.74
5 L Bioreactor	90.22 ± 1.21	4.22 ± 0.25	22.44 ± 0.45	0.45 ± 0.05	0.28 ± 0.03	7.60 ± 0.32	10.95 ± 0.91

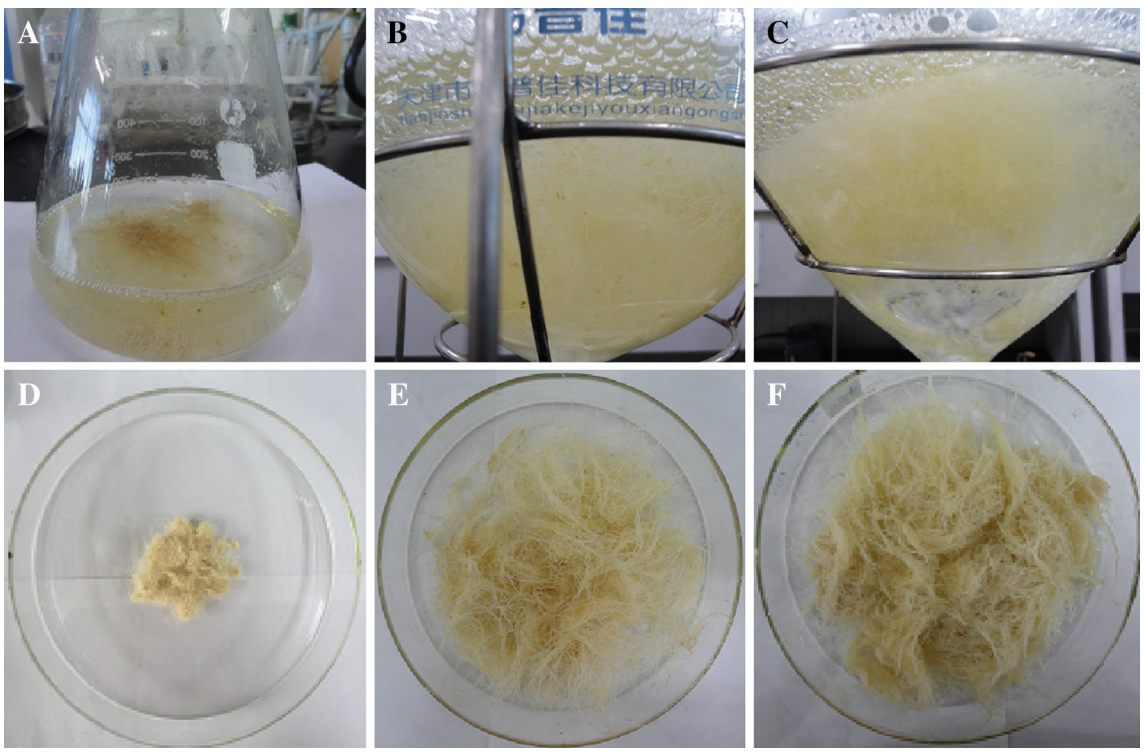

Fig. 1 Scale-up cultures of adventitious roots in a 0.5 L conical flask (**a**), in 3 L (**b**) and 5 L (**c**) balloon-type bubble bioreactors, with harvests of adventitious roots (**d**, **e**) after 30 days' culture

partially supported by *Echinacea purpurea* adventitious roots (Jeong et al. 2009).

Conclusion

In conclusion, the adventitious root culture system of *G. uralensis* was successfully established for the metabolites accumulation. We investigated the effects of nitrogen source and phosphate concentration on root growth and metabolites accumulation. The optimal culture condition we obtained in experiments was an ammonia/nitrate rate of 10:20 and 1.25 mM phosphate concentration. In scale-up culture of adventitious root, the growth rate in 5 L BTBB reached 22.44 which indicated a potential manner for the large-scale production of biomass and bioactive compounds of *G. uralensis*.

Author contribution All authors contributed extensively to the work presented in this paper. Shuangshuang Yin and Yao Zhang designed the experiment and wrote the paper under the guidance of WenYuan Gao. Juan wang and Shuli Man were responsible for viability tests and statistical analysis. Hui Liu was in charge of culturing adventitious roots.

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