Flavonoid Accumulation in Cell Suspension Cultures of *Glycyrrhiza inflata* Batal under Optimizing Conditions

Ying Yang*, Feng He, Longjiang Yu*, Jiaxing Ji, and Yezhen Wang

College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China. Fax: +86–27–87 79 22 65. E-mail: Yulj@hust.edu.cn

* Authors for correspondence and reprint requests

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Cell growth and flavonoid production in cell suspension cultures of *Glycyrrhiza inflata* Batal were investigated under various initial inoculum densities, and sucrose and nitrogen concentrations to develop an optimization method for an improved flavonoid production. Both biomass accumulation and flavonoid production exhibited an "S" curve in one culture cycle, with the greatest value obtained on day 21, which showed that cell growth and flavonoid biosynthesis went along isochronously. Moreover, according to the biomass and flavonoid production, the appreciate inoculum density, and the sucrose and nitrogen concentrations were 50 g FW L^{-1}, 50 g L^{-1} and 120 mmol L^{-1}, respectively. In addition, cell growth and flavonoid production showed a peak of 16.4 g DW L^{-1} and 95.7 mg L^{-1} on day 21 under the optimizing conditions, respectively. The flavonoid productivity of the cells which were cultured for 3 years is higher than that of the 3-year-old plant, which suggested that flavonoid production by cell cultures of *G. inflata* is a potentially profitable method. Therefore, this work is considered to be helpful for efficient large-scale bioprocessing of cell cultures in bioreactors.

Key words: Flavonoid Production, *Glycyrrhiza inflata* Batal, Optimizing Conditions

Introduction

Licorice has extensively been used as a traditional Chinese medicine for over 2000 years. It not only has anti-inflammatory, antibacterial and antiviral activities, but also has immunomodulating, antioxidant and free radical scavenging activities (Shetty *et al.*, 2002). *Glycyrrhiza inflata* Batal, an important specie of licorice, has been frequently used to treat phthisis, contagious hepatitis, ague and gastric diseases (Wang *et al.*, 2004). Through pharmacological studies and clinical practice flavonoids have been demonstrated to carry significant biological or antioxidation activities (Li *et al.*, 1998; Fukai *et al.*, 2002). Usually, it takes at least three years for *G. inflata* plants to be harvested for medicinal purposes. A plant cell culture, a useful method for the production of valuable secondary metabolites, provides an attractive alternative source that can overcome the limitations of extracting useful metabolites from limited natural resources (Thanh *et al.*, 2006; Smolenskaya *et al.*, 2007). It is now widely used as a model system to investigate the production of specific secondary metabolites, because it offers experimental advantages both to basic and applied research and to the development of models with scale-up potential (Buitelaar and Trapmer, 1992).

There were very few studies on cell suspension cultures of *G. inflata* which primarily focused on the study of separation, purification and pharmacology of the flavonoids (Asada *et al.*, 1998; Li *et al.*, 1998, 2000). Moreover, the regulation of the flavonoid biosynthesis in cell suspension cultures of *G. inflata* has not yet been systematically investigated. In plant cell cultures, carbon sources, nitrogen sources, phosphate sources, conditioned medium, inoculum density, and CO_2 accumulation are known to be the main limiting factors. Carbohydrates, especially sucrose, are important carbon and energy sources for most plant cell lines. It has been demonstrated that the initial sucrose concentration can affect a number of culture parameters of secondary metabolites in plant cell cultures such as growth rate and yield (Benavides, 1997; Zhong and Yoshida, 1995; Wang *et al.*, 1997). Consequently, some culture conditions, such as inoculum density, sucrose and nitrogen concentration in the medium, were individually investigated in the present study to develop an optimization method for improved flavonoid production, to ultimately establish a stable cell suspension culture of *G. inflata*.

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Materials and Methods

Plant material and culture conditions for callus induction

The calli were derived from the seeds of *G. inflata* Batal, which were obtained from wild plants growing in the desert of Xinjiang in China, donated by an incorporated company of Xinjiang Kunlunshennong of Northwestern China, and identified by the Planting Center of *Glycyrrhiza* of Xinjiang, China. Seeds were set in a beaker and 98% H_2SO_4 was added to facilitate germination. Then the seeds were cleaned under running tap water and surface-sterilized by 75% ethanol for several minutes. This was followed by two treatments of 10 min rinse in 0.1% mercuric chloride solution and washing with sterile distilled water for five times. After surface-sterilization, the seeds were placed separately in 30 mL Murashige and Skoog (MS) basal medium in 100-mL flasks, and kept in the dark before germination. The cotyledons and hypocotyls were excised when they grew to a length of 3–4 cm, and placed on the surface of MS medium containing 3% sucrose and 0.8% agar supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 1.0 mg L^{-1}), naphthalene acetic acid (NAA, 1.0 mg L^{-1}), and 6-benzyladenine (6-BA, 1.0 mg L^{-1}). All the cultures were maintained at (25 ± 1) °C under 16 h illumination with 60 μmol m^{-2} s^{-1}.

Suspension culture establishment and cell growth determination

The suspension cells were derived from the calli tissue; 5 g calli in a 250-mL flask were subcultured with 80 mL of liquid MS culture medium containing 2,4-D (0.5 mg L^{-1}), NAA (0.5 mg L^{-1}), and 6-BA (0.5 mg L^{-1}) every week until the cells showed continuous and stable accumulation of biomass. Cells of *G. inflata* were cultured in a 250-mL flask with 80 mL of modified liquid MS culture medium at (25 ± 1) °C on a rotatory shaker with a speed of 120 rotations per minute (rpm) under 16 h illumination with 60 μmol m^{-2} s^{-1}. The medium was autoclaved at 121 °C for 20 min.

After cultured in one cycle, the cells were harvested by filtration via a Buchner funnel, washed with distilled water to remove residual medium, and filtered again. Then the weighed fresh cells (FW) were dried at 50 °C to constant dry weight (DW). Cell growth was measured based on the DW.

Assay of flavonoids

The dried pulverized cell samples were weighed (0.10 g exactly) and put into a 10-mL measuring flask. The flavonoids were extracted with 3 mL ethanol/water (70:30, v/v) by ultrasonication for 1 h at 25 °C. After centrifugation at 5500 × *g* for 6 min, the supernatant was extracted three times with EtOAc, then with 95% ethanol. The flavonoid content was the combination of the mass of flavonoids in cells and medium, and was determined by colourimetry according to Zhang *et al.* (2001). Rutin was used as the standard sample.

Statistical analysis

All data are represented as means ± standard errors (SE). Every experiment was repeated at least 3 times. The significant difference between the treatment and the control was statistically evaluated by analysis of variance (ANOVA).

Results and Discussion

Time course of cell growth and flavonoid accumulation

The cell suspension cultures showed continuous and stable accumulation of biomass after 10 subcultures. According to Fig. 1, the time courses of biomass accumulation describe an "S" growth

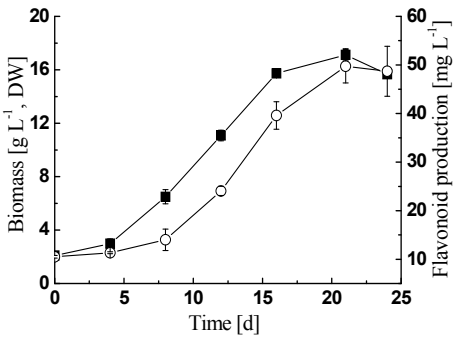

Fig. 1. Time courses of cell growth and flavonoid production in cell suspension cultures of *G. inflata*. The suspension cultures were grown in liquid MS medium containing 0.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} NAA, and 0.5 mg L^{-1} 6-BA. ■ and ○ represent biomass and flavonoid production, respectively. Each data indicates the mean of three independent experiments (mean ± SE).

curve in one culture cycle. Cells grew very slowly during the initial 4 d of cultivation. Thereafter, biomass accumulated rapidly, and reached the greatest value of 17.1 g L^{-1} on day 21. Then the culture entered the stationary phase. Some cultures continued to grow up to day 30, but invariably darkened and appeared less healthy. The flavonoid production increased significantly from the sixth day and reached a peak of 49.7 mg L^{-1} on day 21, then it began to decrease. The results mentioned above indicate that cell growth and flavonoid synthesis went along isochronously in a favourable culture cycle of 21 days.

Effects of inoculum density on cell growth and flavonoid production of cell suspension cultures of G. inflata

The inoculum density is an important factor for plant cell cultures, which can influence not only the cell growth, but also the accumulation of secondary metabolites. Therefore, fresh *G. inflata* cells from 5 to 80 g L^{-1} were transferred into 250-mL flasks. As showed in Fig. 2, the biomass and flavonoid production were low at a low inoculum density of 5 g FW L^{-1}, while they both increased with increasing inoculum density, and reached the peak of 18.2 g DW L^{-1} and 58.7 mg L^{-1} at an inoculum density of 50 g FW L^{-1}, respectively. Further increases in the inoculum density produced a decline in the flavonoid accumulation. These results were found in many plant cell cultures (Wang *et al.*, 1997). A maximum cell biomass of *Perilla frutescens* was obtained at an elevated inoculum density of 50 g FW L^{-1}, and the anthocyanin production was enhanced 23-fold (Zhong and Yoshida, 1995). These results suggested that a higher final cell biomass may be achieved in a cell culture with a higher inoculum density. The mechanisms may include medium composition and culture conditions (Schlatmann *et al.*, 1994). Furthermore, disparity in cell inoculum density could lead to a large difference in culture parameters, for example the concentrations of dissolved oxygen and dissolved gaseous metabolites as well as the related enzyme activities could be altered by accumulated cell biomass. Directly or indirectly, these changes could further affect the cell metabolism. Additionally, it is also possible that initial cell-to-cell communication and/or unknown factors released by inoculum cells are playing a significant role in cell cultures.

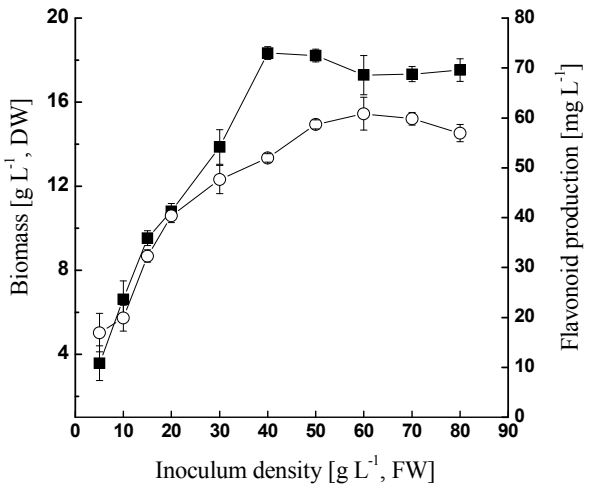

Fig. 2. Biomass and flavonoid accumulation in cell suspension cultures of *G. inflata* inoculated at different inoculum densities. The suspension cultures were grown in MS liquid medium containing 0.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} NAA, and 0.5 mg L^{-1} 6-BA. ■ and ○ represent biomass and flavonoid production, respectively. Each data indicates the mean of three independent experiments (mean ± SE).

Effects of sucrose or nitrogen concentration on cell growth and flavonoid production of cell suspension cultures of G. inflata

Nutrition is required to keep cells growing. As a carbon source, sucrose is used to not only provide the carbon framework of cell essential structural components, but also to offer the energy re-

Table I. The biomass and flavonoid production of cell suspension cultures of *G. inflata* under different sucrose or nitrogen concentrations. The suspension cultures were grown in MS liquid medium containing 0.5 mg L^{-1} 2,4-D, 0.5 mg L^{-1} NAA, and 0.5 mg L^{-1} 6-BA. Each data indicates the mean of three independent experiments (mean ± SE).

Concentration of	Biomass [g L^{-1}]	Flavonoid production [mg L^{-1}]
Sucrose [g L^{-1}]		
10	4.9 ± 0.49	18.8 ± 1.06
50	15.6 ± 0.24	72.3 ± 3.78
Nitrogen [mmol L^{-1}]		
10	9.4 ± 0.62	36.4 ± 1.91
120	13.1 ± 0.56	73.1 ± 2.20

Nitrogen consists of ammonia and nitrate in a ration of 1:1.8.

quired for growth and maintenance. The nitrogen source plays an important role in the synthesis of nucleic acids and proteins. The effects of initial sucrose or nitrogen concentration on growth and flavonoid production of cell suspension cultures of *G. inflata* are shown in Table I. Both the biomass and flavonoid production increased with increasing sucrose or nitrogen concentration, and reached the peak of 15.6 g L^{-1} and 72.3 mg L^{-1} with the sucrose concentration of 50 g L^{-1}, and of 13.1 g L^{-1} and 73.1 mg L^{-1} with the nitrogen concentration of 120 mmol L^{-1}, respectively. In suspension cultures of *Perilla frutescens* for the production of anthocyanin pigments, a relatively high sucrose concentration (45 g L^{-1}) was reported to be favourable for pigment accumulation (Zhong and Yoshida, 1995). Sucrose regarded as a regulator of osmotic pressure, might take an important role concerning the osmotic pressure in the medium. The positive effects of osmotic potential, provoked by high sucrose concentrations in the medium, on the accumulation of anthocyanins have been demonstrated in cell cultures of *Daucus carota* (Rajendran *et al.*, 1992). Thus, it can be speculated that a certain level of osmotic stress caused by a relatively higher initial sucrose concentration may be favourable to product formation, but represses the cell growth.

Cell growth and flavonoid accumulation under the optimizing conditions

The cells were cultured with the sucrose and nitrogen concentration of 50 g L^{-1} and 120 mmol L^{-1}, at an inoculum density of 50 g FW L^{-1}, respectively. The time courses of biomass accumulation and flavonoid production are shown in Fig. 3. From the sixth day, biomass accumulated rapidly and reached the greatest values of 16.4 g L^{-1} on day 21. Flavonoid production also reached a peak

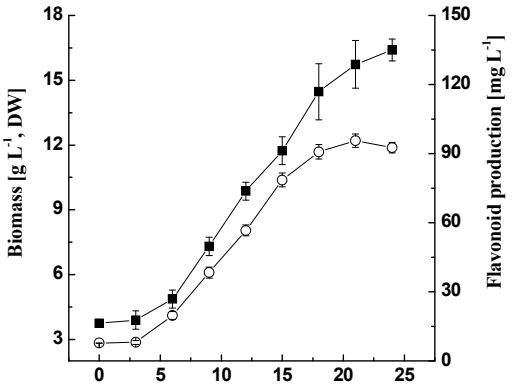

Fig. 3. Cell growth and flavonoid production in cell suspension cultures of *G. inflata* in the optimizing MS medium with sucrose and nitrogen concentrations of 50 g L^{-1} and 120 mmol L^{-1}, respectively, and an inoculum density of 50 g FW L^{-1}. ■ and ○ represent biomass and flavonoid production, respectively. Each data indicates the mean of three independent experiments (mean ± SE).

of 95.7 mg L^{-1} on day 21, which was 1.93 times of the flavonoid production under the initial conditions. The highest content of flavonoids in one cell culture cycle under the optimizing conditions was only 0.6% (about 6 mg g^{-1} DW), which was lower than that of a 3-year-old plant in which the flavonoid content always maintained about 3%. However, it takes at least three years for the *G. inflata* plant to be harvested for medicinal purposes, while the cell growth cycle is only 21 days. Thus, the flavonoid productivity of the cells cultured for 3 years is much more than that of the 3-year-old plant, which suggests that flavonoid production by cell cultures of *G. inflata* is a potentially profitable method. Therefore, this work is considered to be helpful for efficient large-scale bioprocessing of cell cultures in bioreactors.

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