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BRIEF COMMUNICATION

Dynamics analyses of nutrients consumption and flavonoids accumulation in cell suspension culture of *Glycyrrhiza inflata*

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Abstract

The dynamics of biomass accumulation, production of flavonoids and consumption of carbon, nitrogen and phosphate were investigated in *Glycyrrhiza inflata* Batal cell suspensions cultivated in flasks. Biomass accumulation exhibited a "S"-shape curve in each culture cycle, with the greatest values obtained on day 21 (16.4 and 232.4 g dm^{-3} of dry and fresh mass, respectively). Similarly, flavonoids production also got to a peak of 95.7 mg dm^{-3} on day 21. Sucrose was decomposed to reducing sugars which were almost used up on day 22. Nitrate and phosphate in the medium were almost exhausted on day 18 and 10, respectively, while ammonium still maintained at concentration 100 mg dm^{-3} when the cells were harvested. Consequently, the proportion of ammonium to nitrate in the medium should be optimized for higher flavonoid production.

Additional key words: growth, licorice, plant cell culture, secondary metabolites, substrates consumption.

Licorice (*Glycyrrhiza inflata* Batal) has been used as a traditional Chinese medicine extensively for over 2000 years. This species contains flavonoids with significant antioxidative activities (Li *et al.* 1998, Fukai *et al.* 2002). With an increasing demand for flavonoids, the natural sources of *G. inflata* are gradually exhausted. As an alternative approach, plant cell cultures are used for the production of valuable secondary metabolites (Thanh *et al.* 2006, Smolenskaya *et al.* 2007).

There were only few studies on cell suspension cultures of *G. inflata*. Moreover, the regulation of flavonoids biosynthesis, dynamics of product accumulation and substrates consumption have not been systematically investigated. The cell growth and the flavonoids biosynthesis are sensitive to environmental conditions. Consequently, dynamics analysis is required for predicting cell growth and product formation, and for designing cultivation conditions (Takeda *et al.* 1998).

The seeds of *Glycyrrhiza inflata* Batal were obtained from wild plants growing in the desert of Xinjiang in China, donated by Incorporated Company of Xinjiang Kunlunshennong of the Northwestern China, and identified by Planting Center of *Glycyrrhiza* of Xinjiang, China. The seedlings were grown on the Murashige and Skoog (1962; MS) medium containing 3 % sucrose, 0.8 % agar, 1.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 mg dm^{-3} naphthalene acetic acid (NAA) and 1.0 mg dm^{-3} 6-benzyladenine (BA).

The cells were obtained from the callus derived from cotyledons and hypocotyls. They were cultured in 250 cm^3 flasks on a rotatory shaker (a speed of 120 rpm) with 80 cm^3 of modified liquid MS medium containing 3 % sucrose, 0.5 mg dm^{-3} 2,4-D, 0.5 mg dm^{-3} NAA, and 0.5 mg dm^{-3} BA at 25 ± 1 °C, 16-h photoperiod with irradiance of 60 μmol m^{-2} s^{-1}. The media were autoclaved at 121 °C for 20 min, and the pH was adjusted to 5.8 prior to autoclaving.

The cells were harvested by filtration *via* a Buchner funnel, washed with distilled water to remove residual medium, and filtrated again. Then the weighted fresh cells were dried at 50 °C to constant dry mass (d.m.). Cell growth was measured based on fresh mass (f.m.) and d.m.. The flavonoids were extracted with 30 volumes of 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,

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Abbreviations: AA - ascorbic acid; BA - 6-benzyladenine; 2,4-D - 2,4 - dichlorophenoxyacetic acid; DM - dry mass; FM - fresh mass; NAA - naphthalene acetic acid; rpm - rotation per minute.
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then with 95 % ethanol. Flavonoids content were the combination of flavonoids in cells and media, which was determined by colorimetry according to Zhang *et al.* (2001). Rutin was used as the standard sample.

The sucrose and reducing sugar contents in the medium were determined by the resorcin method (Wickremesinhe and Arteca 1993) and dinitrosalicylic acid (DNS) method (Miller 1959), respectively. Additionally, nitrate, ammonium, and inorganic phosphate medium concentrations were assayed by the method of salicylic acid (Hecht and Mohr 1990), ninhydrin (Moore and Stein 1948) and ascorbic acid (Chen *et al.* 1956), respectively.

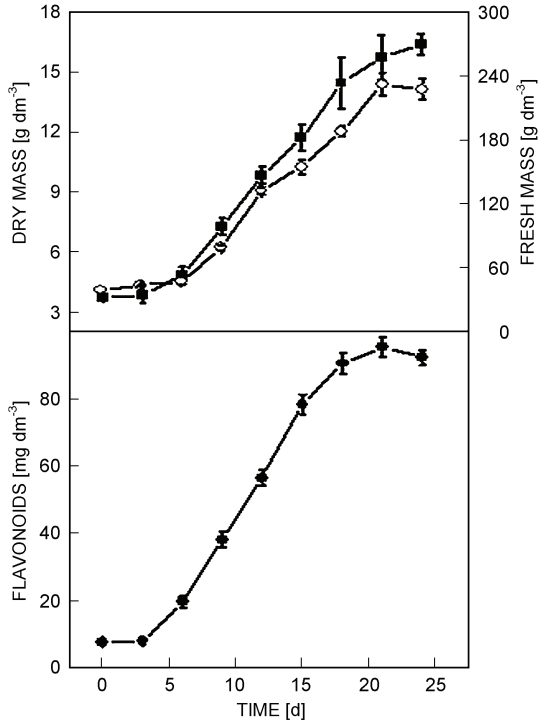

Fig. 1. Time courses of cell growth and flavonoids production in suspension culture of *G. inflata*. The cultures were grown in liquid MS medium containing 0.5 mg dm^{-3} 2,4-D ,0.5 mg dm^{-3} NAA and 0.5 mg dm^{-3} BA. *Closed squares* - dry mass, *open circles* - fresh mass, *closed circles* - flavonoids production. Each point indicated the means ± SE of three independent experiments.

The cell suspension cultures showed continuous and stable accumulation of biomass after 10 subcultures (Fig. 1). In each culture cycle (about 24 d) cells grew very slowly during the initial 6 d of cultivation. Thereafter, biomass accumulated rapidly and reached the greatest values [16.4 g dm^{-3}(d.m.) and 232.4 g dm^{-3}(f.m.)] on day 21. Then the cell culture entered the stationary phase. Some cultures continued to grow up to 30 d, but invariably darkened and appeared less healthy. Flavonoids production increased very slowly at the beginning of the cultivation, but increased significantly from the sixth day and reached a peak of 95.7 mg dm^{-3} on day 21, then began to decrease. The results mentioned above indicated that cells growth and flavonoids synthesis went along isochronously. The highest content of flavonoids in cell culture was 0.6 % was lower than that of 3-year-old plant (3 %). However, taking into account the cost and productivity, we considered that the flavonoids production by the cell cultures of *G. inflata* might be a potentially profitable process.

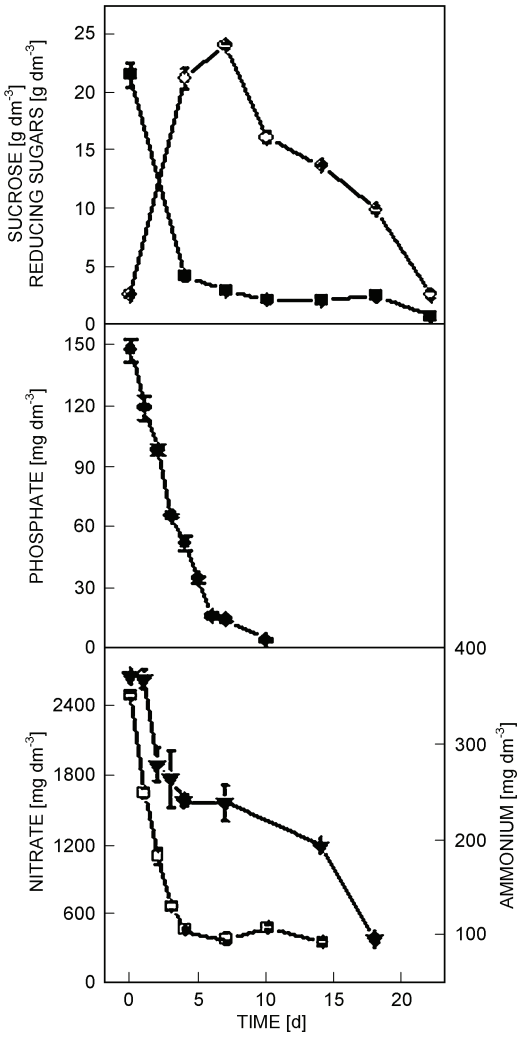

Fig. 2. Time courses of nutrient contents in the medium during cultivation of *G. inflata* suspension cultures. The cultures were grown in liquid MS medium containing 0.5 mg dm^{-3} 2,4-D, 0.5 mg dm^{-3} NAA and 0.5 mg dm^{-3} BA. *Closed squares* - sucrose, *open circles* - reducing sugars, *closed circles* - phosphate, *closed triangles* - nitrate, *open squares* - ammonium. Each point indicated the means ± SE of three independent experiments.

The courses of consumption of sources of carbon, nitrogen and phosphate were also investigated (Fig. 2). Sucrose was used to provide C and energy source. Its concentration decreased sharply during the initial 4 d of cultivation, while content of reducing sugars increased quickly. The results suggested that sucrose had been decomposed immediately to glucose and fructose, which could be consumed directly by the cells (Yamada *et al.*

2003, Shin *et al.* 2003). Reducing sugar concentration reached a peak of 24.03 g dm^{-3} on day 6, which implied that they were used slowly before the sixth day. Thereafter, they were consumed quickly, and almost used up on day 22. The results indicated that carbon consumption was consistent with the cells growth. Sucrose decomposed to glucose and fructose also resulted in an decrease in osmotic potential of the medium, that might affect cell growth and flavonoid biosynthesis.

Nitrogen and phosphate both play an important role in synthesis of nucleic acids and proteins. In order to get the appropriate nutrient combination, time courses of nitrogen and phosphate in the medium were investigated. The results showed that phosphate, nitrate and ammonium all decreased sharply at the initial several days, which suggested the nutrients were taken up fast by the cells (Fig. 2). Phosphate in the medium was exhausted on day 10 at the logarithm growth phase, which was observed in many plants cell cultures (Yamada *et al.* 2003, Shin *et al.* 2003). When the medium phosphate was completely consumed, the cells could utilize intracellular phosphorus stored in the vacuoles to sustain growth (Bieleski and Ferguson 1983). Pepin *et al.* (1995) also proposed that the depletion of phosphate might limit cell division. Therefore, the addition of phosphate after day 10 might facilitate cell division and enhance the biomass accumulation ultimately.

For nitrogen consumption, nitrate decreased in the medium and was almost run out of on day 18. Although the ammonium was used quickly at the first 4 d, it still maintained at concentration 100 mg dm^{-3} (which was 28.5 % of the initial concentration) when the cells were harvested. Most plant cells utilize ammonium firstly and nitrate later (Shin *et al.* 2003). When the ammonium concentration in the medium is low, most of the ammonium is metabolized by the cells, while in the case where the ammonium concentration is too high, only a small part can be metabolized and the excess has inhibitory effects on the cell metabolism (Bensaddek *et al.* 2001). Another consequence of the maintenance of ammonium could be a direct or indirect repressive effect on the nitrate assimilation (Crawford 1995). Thus, the ammonium consumption was faster than nitrate at the beginning of the cultivation. But ammonium uptake decreased in the presence of nitrate (Dortch and Conway 1984), which might be one of the reasons of ammonium maintaining till the end of culture. Therefore, it was considered that the proportion of ammonium to nitrate in the medium should be optimized.

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