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Effect of Initial Phosphate Concentration on Cell Growth and Ginsenoside Saponin Production by Suspended Cultures of *Panax notoginseng*

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ABSTRACT

Effects of initial phosphate concentration on the growth, ginsenoside saponin production, and the consumption of sugar and nitrogen sources by suspended cells of *Panax notoginseng* (Burk) F. H. Chen were investigated in a 250-mL shake flask. The results indicate that by increasing the initial phosphate concentration in the medium in the range of 0–1.25 m*M*, both the cell growth and the saponin accumulation were greatly improved, and the utilization of sugar and nitrogen sources was also increased. The highest production, productivity, and yield of ginsenosides obtained were 0.98 g/L, 45.5 mg/L/d, and 0.030 g/g at 1.25 m*M* of initial medium phosphate. At a relatively higher level of medium phosphate, i.e., 2.0 m*M*, the product accumulation was inhibited to some degree, although the cell growth was not.

Index Entries: Cell growth; ginsenoside saponin; initial phosphate concentration; nutrient utilization; *Panax notoginseng*; plant cell culture; secondary metabolite production.

INTRODUCTION

Recently, plant cell culture has received much attention for production of useful plant-specific chemicals. This is because:

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1. Plants produce more than 20,000 types of chemicals, including pharmaceuticals, pigments, and other fine chemicals, which is four times more than those obtained from microbes;
2. Some of these chemicals are difficult to synthesize chemically, or it is difficult to produce or to increase the amounts produced by microorganisms through genetic engineering; and
3. Plant cell culture is not limited by environmental, ecological, or climatic conditions, and cells can thus proliferate at higher growth rates than whole plants in cultivation.

Panax notoginseng, which belongs to the Araliaceae family, is one of the most famous traditional Chinese medicinal plants, and is mainly distributed in the Yunnan and Guangxi Provinces, China. Its hemostatic qualities and abilities to promote blood circulation, relieve pain, and cure bleeding wounds and trauma are recorded in traditional Materia Medica (*1*). The major compounds of pharmaceutical importance in the plant have been isolated and identified to be ginsenoside saponins (*2–4*), and they recently been used for treatment of diseases in cardiovascular, cerebrovascular, central nervous, as well as blood and hematopoietic systems, and so on. The supply of wild *P. notoginseng* has run short for a long time, and the field cultivation of the plant is rather complicated. Production of its pharmacologically active metabolites (ginsenosides) by plant tissue and cell culture may be a cost-effective approach to meeting the popular market demand.

Compared with numerous publications concerning with in vitro culture of *Panax ginseng* (*5–7*), there have been very few papers on the cell cultures of *P. notoginseng* as reported by Zheng and Wang (*8*), and Zhou et al. (*9*). Those studies are mainly concerned with the influence of phytohormones, inoculum densities, and aeration rates on suspended notoginseng cultures. Although the initial phosphate level in medium was claimed to be one of the most important factors influencing plant cell cultures (*10–14*), its effect on suspended ginseng cultures was not well understood. In this work, we investigated the effect of initial medium phosphate concentration (P_i) on the cell growth and ginsenoside saponin production by suspended cultures of *P. notoginseng* (Burk) F. H. Chen to provide an insight into the regulation of the cell growth and metabolite biosynthesis by P_i. The results indicate that the phosphate concentration is a significant factor affecting both the growth and secondary metabolism in the suspension cultures of notoginseng cells.

MATERIALS AND METHODS

Plant Cells, Medium, and Culture Conditions

Suspended cells of *P. notoginseng*, which were induced from the cultivated root, were maintained in Murashige and Skoog medium supple-

mented with 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.7 mg/L of kinetin, and 30 g/L of sucrose. The cells were subcultured every 13–15 d in a 250-mL conical flask with a working volume of 50–mL on a rotary shaker Model HZ-81 (Shanghai Yuejin Medical Instrument Factory, Shanghai) in darkness at a rotation speed of 110 rpm and culture temperature of 25°C. The medium pH was adjusted to 5.8 before autoclaving. The inoculation size was controlled at 25.4 g wet cells/L, i.e., 1.53 g dry cells/L.

Analytical Procedures

Residual sugar concentration in medium was determined as described previously (*15*). Ammonium and nitrate concentrations in the medium were measured by the method of phenol-hypochlorite reaction (*16*) and by an ion-selective electrode PNO_3-1 (Shanghai Dianguang Device Factory, Shanghai), respectively. Inorganic phosphate concentration in the medium was determined according to the ascorbic acid method (*17*).

Dry cell mass was determined as follows. The cells of a sample were washed with a large amount of distilled water, centrifuged at 2500 rpm for 10 min, and then separated from supernatant. The cells were weighed after dried to constant weight at a temperature below 50°C for several days.

For ginseng saponin assay, a 200-mg aliquot of the dried cell powder was soaked in *n*-BuOH at 4°C for 1 d, and then broken for 30 min by ultrasonic treatment. The saponin content was determined by TLC-colorimetric analysis as reported (*4,6,9*). The authentic ginsenoside was purchased from the Drugs and Biological Products Identification Institute of China (Beijing).

Calculation of Cell Growth Rate and Yields of Cells and Saponins

Because the intrinsic exponential growth phase in batch culture of the plant cells is very short (unpublished results), the cell growth is evaluated in terms of the cell growth rate (GR) instead of the specific growth rate. Supposing the initial cell viability was 100%, the value of GR was calculated by dividing the cell dry weight increased by the initial cell mass (i.e., 1.53 g/L by dry wt) and by the cultivation time (days) (*18*), i.e.:

$$GR = [(\text{final cell mass}) - (\text{initial cell mass})] / (\text{initial cell mass}) / (\text{time}) \quad (1)$$

The observed yield coefficients of cells and ginsenoside saponins in the cell cultures were calculated according to the following formulae (*19*):

$$Y_{x/s} = \delta X / \delta S \quad (2)$$

$$Y_{p/s} = \delta P / \delta S \quad (3)$$

where X represents the cell mass, and S the substrate (sucrose); δX is the mass of cells increased, and δS the sucrose consumed. P represents the product (saponins), and δP is the saponins produced by the cells.

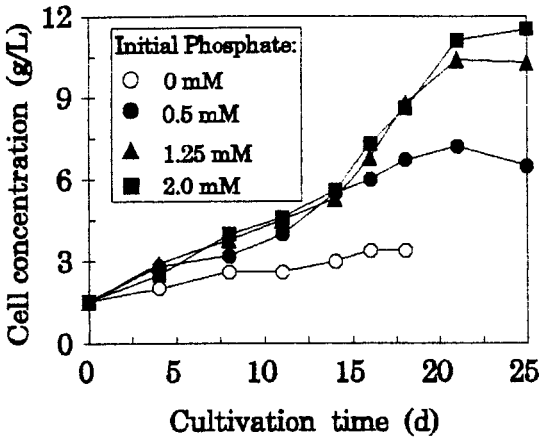

Fig. 1. Time profiles of the growth of suspended cells of *Panax notoginseng* in a 250-mL conical flask culture at a different initial phosphate concentration.

Table 1
Effects of P_i on the Utilization of Sugar and Nitrogen Sources, GR, and Productivity of Ginsenoside Saponins in Suspended Cultures of *Panax notoginseng* Cells in a 250-mL Conical Flask

Initital phosphate concn., m*M*	Utilization, %			GR, l/d	Ginsenoside productivity, mg/L/d
	Sugar	NH_4^+	NO_3^-		
0	31.8	15.0	18.8	0.076	6.7
0.5	80.3	71.7	74.3	0.176	29.8
1.25	97.6	81.7	70.5	0.276	45.5
2.0	97	83.3	86.3	0.298	41.7

RESULTS AND DISCUSSION

Effect of P_i on the Cell Growth

In the experiments, four levels of P_i, i.e., 0, 0.5, 1.25, and 2.0 m*M*, were used for the investigation. Figure 1 shows the growth profiles of the suspended cells of *P. notoginseng* at a different phosphate level in a 250-mL shake-flask culture. The final cell concentration obtained was significantly increased by increasing the initial phosphate level in the range of 0–1.25 m*M*, and continued to increase slightly with a further increase of initial medium phosphate from 1.25 to 2.0 m*M*. The cell growth rate (GR) was 0.076, 0.176, 0.276, and 0.298 (l/d) at 0, 0.5, 1.25, and 2.0 m*M* of P_i, respectively (Table 1). Similar phenomena were also reported in the cases of *Hyoscyamus muticus* hairy root culture (*10*) and *Holarrhena antidysenterica* suspension culture (*12*), but in the hairy root culture of *Datura stramonium*,

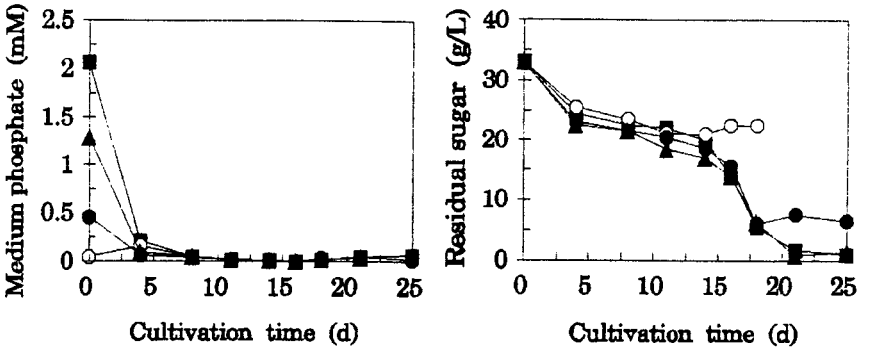

Fig. 2. Changes in medium phosphate level and residual sugar concentration during suspended cultivation of *P. notoginseng* cells in a shake flask at a different initial phosphate concentration. The symbols are the same as those in Fig. 1.

the root growth was the best at 1 m*M* of initial medium phosphate, and it was inhibited at a greater or lesser level of phosphate in medium (*13*).

Effect of P_i on Nutrient Consumption

The dynamic changes in medium phosphate level and residual sugar concentration during suspension cultivation of *P. notoginseng* at the different initial phosphate concentrations are shown in Fig. 2. A rapid decrease in the medium phosphate levels during first 4 d of cultivation was observed, and the phosphate concentration in the medium was almost zero after the eighth day of cultivation. Obviously, the cell growth was not in parallel with the phosphate consumption, and the cultured cells still continued to grow even after the depletion of medium phosphate (cf. Fig. 1). The reason may be that the cells utilize intracellular phosphorus to sustain its continuing growth and metabolic synthesis (*12*), and our preliminary data also supports this consideration (unpublished results).

The patterns of changes in residual sugar concentration during the cell cultivation coincided well with the corresponding growth curves for each case (cf. Figs. 1 and 2). The final sugar concentration was still at a high level of about 2% (w/v) in the cultivation without medium phosphate, whereas a little sugar (6.4 g/L) remained at the initial phosphate concentration of 0.5 m*M*, and the sugar was almost completely consumed at 1.25 or 2.0 m*M* of initial phosphate in the cell cultures. The time profiles of ammonium and nitrate concentrations in the medium during the notoginseng cultivations at the different initial phosphate levels were also investigated, and the same phenomena as the changes in residual sugar concentration were observed for each case (data not shown). As shown in Table 1, with the increase of P_i in the range of 0–2.0 m*M*, the utilization of sugar and nitrogen sources was steadily increased. The effect of P_i on the consumption of nitrogen sources was not reported in other cases of plant cell cultures (*10–13*).

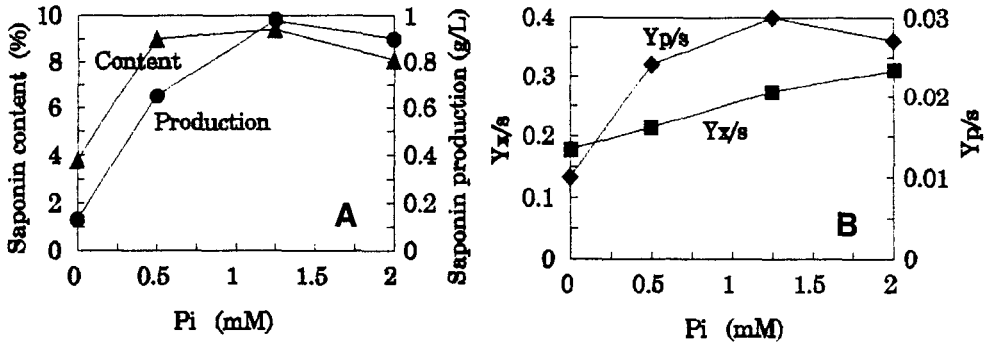

Fig. 3. Effects of P_i on the saponin content and production (**A**), as well as yields of cells ($Y_{x/s}$) and saponins ($Y_{p/s}$) (**B**) in cell cultures of *P. notoginseng* in a shake flask.

Effect of P_i on the Production and Yield of Saponins

Figure 3A shows the effects of P_i on the content and production of ginsenoside saponins in the cell cultures of *P. notoginseng* in a shake flask. The highest saponin production, 0.98 g/L, and its specific production (i.e., saponin content), 9.4%, were reached at 1.25 m*M* of P_i. At a relatively higher level of initial phosphate, i.e., 2.0 m*M*, both the saponin production and its specific production were decreased to some degree. As shown in Fig. 3B, the maximum product yield ($Y_{p/s}$) obtained in the notoginseng culture was 0.030 g/g at the P_i level of 1.25 m*M*, whereas the cell yield ($Y_{x/s}$) reached the highest, i.e., 0.311 g/g, at 2.0 m*M* of P_i. In addition, a relatively high saponin productivity of 45.5 mg/L/d was attained at 1.25 m*M* of P_i (Table 1). In the case of suspended cultivation of *H. antidysenterica* cells for production of a steroid alkaloid conessine, Panda et al. (*12*) claimed that the alkaloid production and its specific production were optimal at phosphate concentration of 0.25 m*M*. In hairy root cultures of *D. stramonium* for hyoscyamine production, it was reported that the alkaloid accumulation was the highest at 0.5 m*M* of initial medium phosphate, and the production was significantly reduced at a phosphate level over 5 m*M* (*13*). All the above information indicates that a detailed investigation of the effect of initial phosphate level for each case is very necessary for the efficient production of secondary metabolites in plant cell and tissue cultures.

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