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Effects of Phosphate, Precursor and Exogenous Berberine on the Production of Alkaloid in Plant Cell Cultures

KIM, DONG-IL*

MOGAM Biotechnology Research Institute,
341 Bojung-Ri, Goosung-Myun, Yongin-Goon, Kyonggi-Do 449-910, Korea

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The effects of phosphate concentration in the medium, feeding of biosynthetic precursor, and the addition of exogenous berberine on cell growth and berberine production were studied in cell suspension cultures of *Thalictrum rugosum*. The depletion of phosphate in the medium enhanced the specific productivity up to twofold with significant release of berberine into the medium. Extracellular berberine was 19% of the total in the culture without phosphate while it was 2-5% of total berberine in the culture with even low amounts of phosphate. Precursor feeding was not effective in enhancing alkaloid formation. Initial presence of exogenous berberine did not have much effect on cell growth and alkaloid production. It was found that the cells have the capacity to take up large quantities of berberine. When 500 mg·l^{-1} of berberine was added exogenously at the beginning, 81% of total berberine was found in the cells.

Higher plants produce a variety of important chemicals and there is a continuing interest in cell suspension as a controllable source of these compounds (1, 23). This could provide for continuous and reliable manufacture of industrially important chemicals from higher plants in any locations (2, 6).

In order to develop a successful process for production of a secondary metabolite, a series of studies are required on factors affecting production yield and/or productivity (18). Once high-producing cell lines are selected, optimization of process variables is a next step to increase production levels even higher. The culture conditions to be optimized for growth and production are nutrition (carbohydrate, nitrogen, phosphate sources, mineral salts and their concentrations), vitamins, quality and quantity of growth regulators, irradiation, temperature, pH, aeration, shear strength, and the addition of biosynthetic precursors. The composition of the medium is extremely important for cell growth and the expression of the biosynthetic pathways leading to secondary metabolites. However, in the absence of a unifying theory, media optimization in plant cell cultures is currently done purely on an empirical basis (21).

*Corresponding author
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There have been numerous studies on the addition of biosynthetic precursors to plant cultures which produce secondary metabolites. Sometimes, the addition resulted in an increase of product level (15, 17, 20), but negative or no effects have also been reported (7, 19). These controversial reports explain that there is no general effect of precursor feeding and this has to be studied case by case. A major stumbling block in attempts at "diagnostic biochemical manipulation" is again our comparative lack of knowledge of the biosynthetic pathways for many of the secondary products (10).

Berberine is a quaternary isoquinoline alkaloid found in several plant species and its biosynthetic pathway is well known. It has been used as an antibacterial, antimalarial, and as a stomach drug in the Orient. In addition to its clinical use, berberine is also used as a fluorescent marker in several areas of medical research. Many attempts have been made to produce berberine in callus or cell suspension cultures (16, 22), mostly in Japan, and now it is being produced industrially (13).

Gould and Murashige (14) examined the physiological effects of exogenous berberine in callus and shoot growth of both *Nandida domestica* culture which produced berberine and *Nicotiana tabacum* culture which could not synthesize berberine. They reported that the growth of *Nandida* culture was stimulated at concentrations as

high as 100 mg·l^{-1} and that a low concentration stimulated *Nicotiana* growth, but growth was repressed in concentrations above 10 mg·l^{-1}. Recently, the toxicity of *Cinchona* alkaloids to cell cultures of *C. ledgeriana* in relation to alkaloid uptake and the possibility for selecting high-yielding cell lines has been studied (24).

In this study, effects of phosphate concentration in the medium, precursor feeding, and the addition of exogenous berberine on the production of berberine were investigated in suspension cultures of *Thalictrum rugosum*. The uptake of berberine by cells is also discussed.

MATERIALS AND METHODS

Plant Cell Cultures and Culture Medium

The cell suspension culture of *Thalictrum rugosum* was kindly provided by Dr. Peter Brodelius (University of Lund, Lund, Sweden) and has been maintained on Murashige and Skoog (MS) medium prepared from MS salt mxiture (GIBCO laboratories, Grand Island, NY, USA) with the addition of 2 μM of 2,4-dichlorophenoxyacetic acid (2,4-D), vitamin stock solution and 30 g·l^{-1} of sucrose as carbon source. The pH was adjusted to 6.0 with 1 N KOH (3, 4). The suspension cultures were grown in 125 m*l* Erlenmeyer flasks with 50 m*l* of medium on a gyrotory shaker (Model G10, New Brunswick Scientific Co., Inc., Edison, NJ, USA) at 200 rpm. The temperature of the culture room was 25℃ and cultures were exposed to 18 h of cool white fluorescent light per day. Subcultures have been done weekly by 1:3 dilution.

Batch Experiment Procedures

For the examination of the effects of phosphate, precursor, and exogenous berberine in shake flasks, cells in the late exponential growth phase, which are usually 5-6 days old, have been used. To avoid heterogeneity of the inoculum, all the cells from different flasks were collected in autoclaved large flask and mixed well by shaking. The cells were filtered through Whatman No. 1 filter paper on a Buchner funnel under slight vacuum and washed with fresh medium which was prepared depending on the purpose of the experiment. As fresh weight, 5g of cells were inoculated into a 125 m*l* Erlenmeyer flask containing 50 m*l* of medium. Two or three replicas of flasks were sacrificed for samples. Two separate experiments were performed for tyrosine feeding. Tyrosine was added at the 7th day for one experiment and at the 10th day for the other experiment. After filtration, the cells were collected for cell mass measurement and intracellular product determination. The filtrates were assayed for extracellular product.

Growth Measurement

The cell suspensions were filtered and washed with distilled water, then dried in an oven at 60℃ to constant weight to determine the dry cell weight (DCW).

Alkaloid Analysis

The samples for intracellular berberine analysis were collected by taking 0.5g of cells by fresh weight. To each sample, 20 m*l* of HPLC grade methanol was added and the suspension was sonicated at 125 W for 10 min. The filtrates obtained during the cell mass measurement were collected for the analysis of extracellular berberine in the medium. A filtered sample (10 μl) was injected into the HPLC system with a UV detector (Kratos Corp., Ramsey, NJ, USA). A SUPELCOSIL LC-18-DB column was used with a Supelco LC-18 precolumn. The mobile phase contained 1 mM tetrabutylammonium phosphate in water adjusted to pH 2 with phosphoric acid (60%) and acetonitrile (40%). The flow rate was 2 m*l*·min^{-1} and the measuring UV wavelength was 265 nm.

RESULTS AND DISCUSSION

Effects of Phosphate Concentration

With earlier experiments, we found that the concentration of phosphate in the medium was very important both for cell growth and product formation. In addition to carbon and nitrogen sources, phosphate is one of the key substrates controlling cell metabolism. As the normal concentration of phosphate in MS medium is 1.75 mM, a wide range of concentration up to 20 mM was tested to examine the response and to arrive at an optimum condition. The results are illustrated in Fig. 1. Interestingly, the control concentration of 1.75 mM was a critical point for both growth and berberine production. In the concentration between 1.75 and 20 mM, cell mass and product level were almost constant. Also, concentrations below 1.75 mM brought about a sharp variation. The final cell mass was reduced probably due

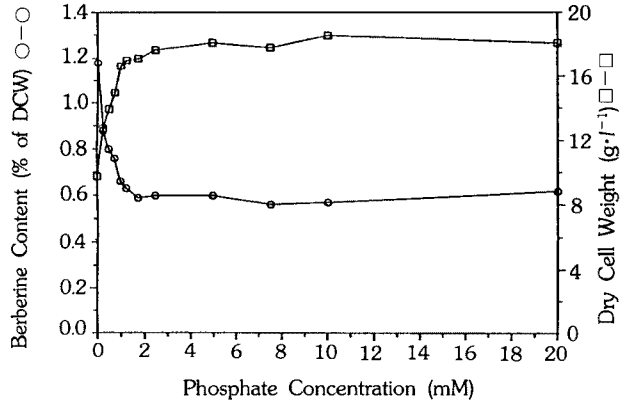

Fig. 1. Cell growth and berberine production at various concentrations of phosphate in the media.

to phosphate depletion whereas specific berberine production increased as phosphate concentration got lower. When the phosphate was removed from the medium, the specific production was the highest while cell growth was very low. Slight cell growth without phosphate may be due to a trace amount of phosphate carried over. The specific content of berberine in the culture without phosphate was almost twice as that in the control culture while cell mass was only one-half of the control. There are many examples that show secondary metabolites can be produced at higher levels by reducing the concentration of the main medium components; particularly, nitrate, ammonium, and phosphate (12). Fujita *et al.* (11) demonstrated that the yield of cells increased with an increase in the concentration of phoshate below 0.24 mM, but became independent of concentration above 0.24 mM. This is somewhat lower compared to our results.

In addition to the increase of specific productivity in the medium without phosphate, the proportion of extracellular product was higher than any other culture with phosphate. Extracellular berberine was 19% of the total in the culture without phosphate while it was 2-5% of total berberine in the cultures with even low amounts of phosphate. This suggests that phosphate deficiency has a relation to the release of berberine into the medium. Very recently, Berlin *et al.* (4) reported that cell cultures of *T. rugosum* released their protoberberine alkaloids into the medium when cells were transferred to fresh medium lacking phosphate. Their results on the concentration of phosphate between 0-2.4 mM showed nearly the same pattern on cell growth and alkaloids specific yields as those described above.

To exploit all these results for biotechnological purposes, a well organized strategy is necessary for optimal production of berberine and its purification. Decoupling of growth and product formation may require two stage culture. Product release without changing viability is an another noteworthy phenomenon.

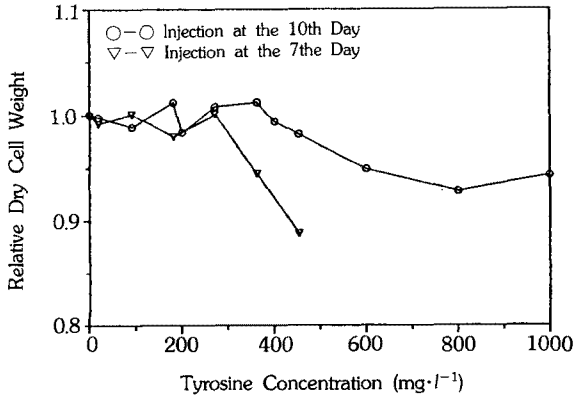

Fig. 2. Effect of tyrosine feeding on cell growth.

Effects of Precursor Feeding

Tyrosine is a precursor of berberine and the effect of its addition was investigated in concentrations up to 5 mM. The effects on growth and berberine production are shown in Fig. 2 and Fig. 3. As expected, the presence of high concentration of tyrosine was toxic to the cells causing cell death and concomitant cell lysis which induced the release of berberine into the medium. Below 1 mM (181.2 mg·l^{-1}), the effect was not significant with respect to both cell growth and berberine production. At high concentration, berberine production was increased in spite of a reduction seen for the cell mass. This increase does not seem to originate from the incorporation of precursor into the biosynthetic pathway. In summarizing the results, the effects of tyrosine was found to be negligible. The 25% increase in berberine level at high concentration of tyrosine may be because of the stress to the cells, not because of the incorporation of precursors. In this experiment, tyrosine was fed at the end of the growth phase in order not to affect cell growth. However, the timing for feeding may be very

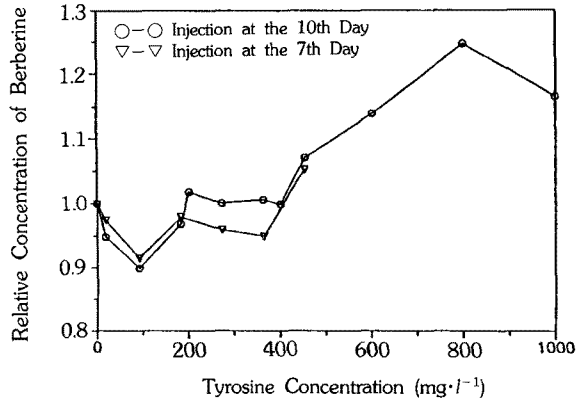

Fig. 3. Effect of tyrosine feeding on berberine production.

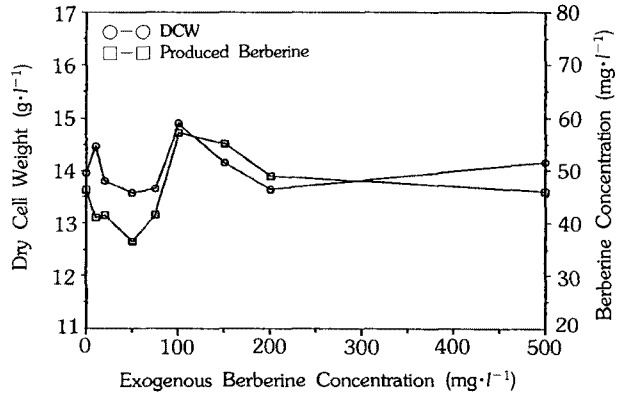

Fig. 4. Effect of exogenous berberine on the production of berberine and cell growth.

important and should be dealt with in more detail separately.

Effects of Exogenous Berberine

In order to study the effects on cell growth and berberine production as well as its uptake, berberine was added exogenously into cell suspension culture. When the product concentration is high in most systems, cell growth may be inhibited and product formation can be feedback-inhibited or repressed. The influences of exogenous berberine on growth and berberine production are illustrated in Fig. 4. Initial presence of exogenous berberine in the medium did not affect the cell growth greatly and there was no indication of cell growth inhibition at all in the concentration range tested. What was more, cell growth was slightly better than that of the control culture at a concentration of 100 $mg \cdot l^{-1}$. Due to the growth-associated nature of product formation, berberine concentration produced by cells showed almost the same trend as the cell growth did. Therefore, it was clear that there was no inhibition or repression of growth by product with a concentration as high as 500 $mg \cdot l^{-1}$ of berberine. Here again, the actual production of berberine was the best when 100 $mg \cdot l^{-1}$ of berberine was added exogenously in the beginning. On top of these findings, the most interesting phenomenon was the berberine uptake by the cells.

The alkaloid usually accumulated in vacuoles. A specific transport mechanism may be responsible for the selective uptake of alkaloids into the vacuoles. This mechanism mediates the accumulation of natural compounds within the cell and may influence the productivity. Deus-Newmann and Zenk (8) showed alkaloid compartmentation in vacuoles and the accumulation against a concentration gradient. The involvement of pH in the release of alkaloids has been described (5) and the vacuolar transport mechanism was studied (9). For easier harvest of products from a biotechnological point of view, it would be desirable to make cells release products into the medium without reducing cell viability and product yield. Berlin *et al.* (4) recently analyzed the nutritional factors required and the impact of physiological state for alkaloid excretion by cell cultures of *T. rugosum* which is the same cell line used in this study.

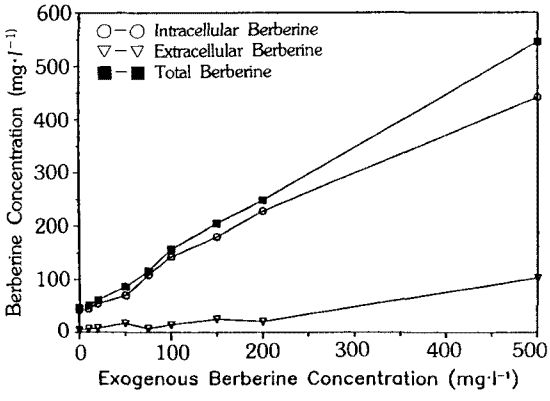

Fig. 5. Distribution of berberine after 8 days culture with exogenous berberine.

As can be seen in the distribution characteristics shown in Fig. 5, most of berberine was found in the cells after the end of culture. This means that most of berberine added exogenously was taken up by cells. When 500 $mg \cdot l^{-1}$ of berberine was added exogenously in the beginning, 81% of the berberine was found in the cells and berberine % of dry cell weight was 3.12%. This tells us that the cells have the capacity to take up large quantities of berberine and may accumulate most of the product intracellularly if we can select high producers. The higher the concentration of added berberine, the more the berberine remaining in the medium even though plenty of berberine was taken up by cells.

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