식물조직배양학회지 제22권 제4호
Korean J. Plant Tissue Culture
Vol. 22, No. 4, 183~187(1995)

Regulation of Growth and Catharanthine Production by the Intracellular Phosphate Level in Hairy Root Cultures of *Catharanthus roseus*

Kyung-Hee JUNG, Sang-Soo KWAK, Cha Yong Choi[1], and Jang R. LIU*
Bioresources Research Group, Korea Research Institute of Bioscience and Biotechnology, KIST, P.O. Box 115, Yusong, Taejon, 305-600; and [1]Department of Chemical Technology, Seoul National University, Seoul, 151-742. *Corresponding author.

세포내 인산농도에 의한 일일초 모상근생장 및 Catharanthine 생산의 조절

정경희 · 곽상수 · 최차용[1] · 유장렬*
한국과학기술연구원 생명공학연구소 생물자원연구그룹, [1]서울대학교 공업화학과

The relationship between the intracellular phosphate level and catharanthine production in hairy root cultures of *Catharanthus roseus* was investigated. The growth of hairy roots increased in proportion to the phosphate concentration in the growth medium. When hairy roots were cultured in a phosphate-free medium, the catharanthine content was increased to the highest level. As the phosphate concentration in the medium was enhanced, the catharanthine content decreased proportionally. Hairy root cultures with elevated intracellular phosphate concentrations indicated that growth was proportional to the level of intracellular phosphate. Catharanthine production increased abruptly below an intracellular phosphate level of 100 μmoles/g dry wt. The intracellular phosphate level may play a key role in the regulation of growth and secondary metabolite production.

Key words: phosphate-free medium, secondary metabolite production

The effects of nitrogen, phosphate, and other inorganic salt components in the medium on indole alkaloid production of *Catharanthus roseus* cell cultures have been extensively investigated (Ganaphati et al., 1990). Plant cell suspension cultures are often characterized by a rapid increase in biomass, but levels of secondary metabolite production are generally low (Zenk et al., 1977). In many cases the use of growth limiting conditions may lead to an increased accumulation of secondary metabolites. Knobloch and Berlin (1980) have found that a high concentration of sucrose is the only prerequisite for indole alkaloid production in *C. roseus* cell cultures, and that addition of phosphate at a minimum level to the medium stops production. Toivonen et al. (1991) have reported contradictory results concerning the effect of phosphate on the growth of *C. roseus* hairy roots and indole alkaloid production, in which the specific production of alkaloid is greatest at the lowest phosphate level. When *C. roseus* hairy roots were cultured in a phosphate-free medium, the catharanthine content increased to its highest level of 3.5 mg/g dry wt (Jung et al., 1994). However, it remains to be determined whether the intracellular phosphate level per se regulates growth and indole alkaloid production. In this study to elucidate the role of intracellular phosphate in growth and indole alkaloid production in *C. roseus* hairy root cultures, the growth kinetics and phosphate uptake of hairy roots are described and the relationship between the intracellular phosphate level and indole alkaloid production is examined.

MATERIALS AND METHODS

Plant Material

Hairy root clone LB1 of *Catharanthus roseus* (L.) G. Don cv Little Bright Eye, induced by infection with *Agrobacterium rhizogenes* strain 15834 and subsequently selected for and high yield of catharanthine (Jung et al., 1992) was used.

Culture Conditions of Hairy Roots

Approximately 0.5 g fresh wt of hairy roots was inoculated into 50 mL of 1/3 SH (Schenk and Hildebrandt, 1972) medium in a 250 mL Erlenmeyer flask, and subcultured every 2 weeks. To investigate the effects of phosphate, hairy roots were cultured in 1/3 SH media containing 0, 0.65, 1.3, 2.6, and 5.2 mM phosphate. To elucidate the effects of intracellular phosphate on growth and alkaloid production, hairy roots with elevated cellular phosphate concentrations were prepared by addition of potassium phosphate (KH_2PO_4) to 1/3 SH medium. Phosphate-enriched hairy roots (80, 180, and 340 μM/g dry wt) were inoculated into phosphate-free 1/3 SH medium. All cultures were maintained in the dark at 25°C on a gyratory shaker (100 rpm).

Analytical Methods

Hairy roots were harvested and washed with double-distilled water before measuring the fresh wt. After drying at 60°C for 24 h, the dried biomass was used for measurement of dry wt and the intracellular phosphate level.

Analysis of indole alkaloids was performed according to the method of Jung et al. (1992). For the quantitative analysis of catharanthine, alkaloid extracts were loaded onto a Bondapak C_{18} column (3.9 × 300 mm) connected to a Spectra-Physics HPLC system. Indole alkaloids were eluted using a solvent mixture of methanol, acetonitrile, and 5 mM diammonium hydrogen phosphate (3/4/3, v/v/v, pH 7.3) at a flow rate of 1 mL/min, and detected at 298 nm.

The level of inorganic phosphate was determined by the colourimetric method of Chen et al. (1956). To measure the intracellular phosphate level, homogenized hairy roots in distilled water were frozen and thawed to rupture the cells. The sample was centrifuged at 3000 rpm for 10 min, then the supernatant was analysed. A reagent solution of 2.1 mL (10% ascorbic acid and 0.42 mM ammonium molybdate in H_2SO_4, v/v, 1:6) was added to 0.9 ml of appropriately diluted sample solution, and the mixture was incubated at 45°C for 20 min. Absorbance of the developed colour was measured at 820 nm.

RESULTS AND DISCUSSION

When hairy roots were cultured in 1/3 SH medium containing 0, 0.65, 1.3, 2.6, and 5.2 mM phosphate, increased levels of the phosphate concentration up to 2.6 mM enhanced the growth of the hairy roots (Fig. 1A). However, higher concentrations of phosphate (2.6 and 5.2 mM) extended the lag phase and decreased the growth rate of the hairy roots. The catharanthine content of hairy roots varied drastically from the phosphate concentration (Fig. 1B). When hairy roots were cultured in media with initial phosphate concentrations below 0.65 mM, the catharanthine content increased steadily during the culture period. However, as the phosphate concentration increased the catharanthine content decreased proportionally. After 21 days of culture, an opposite response direction in growth and catharanthine production to the phosphate concentration in the medium was evident. Inhibition of secondary metabolite production and stimulation of cell growth by high phosphate concentrations have also been reported (Toivonen et al., 1991; Knobloch and Berlin, 1980).

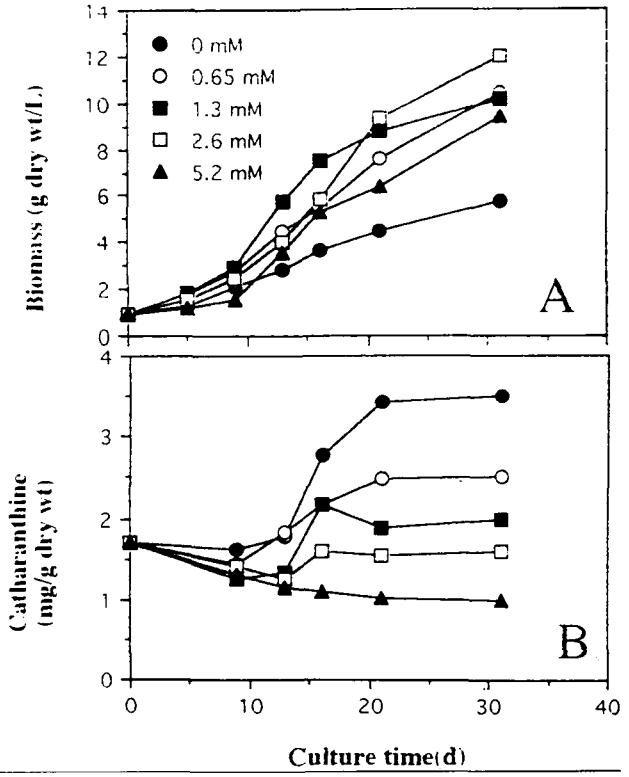

Figure 1. Profiles of growth and catharanthine content of hairy roots culture in medium containing various concentrations of potassium phosphate (KH_2PO_4) during culture period (A, B).

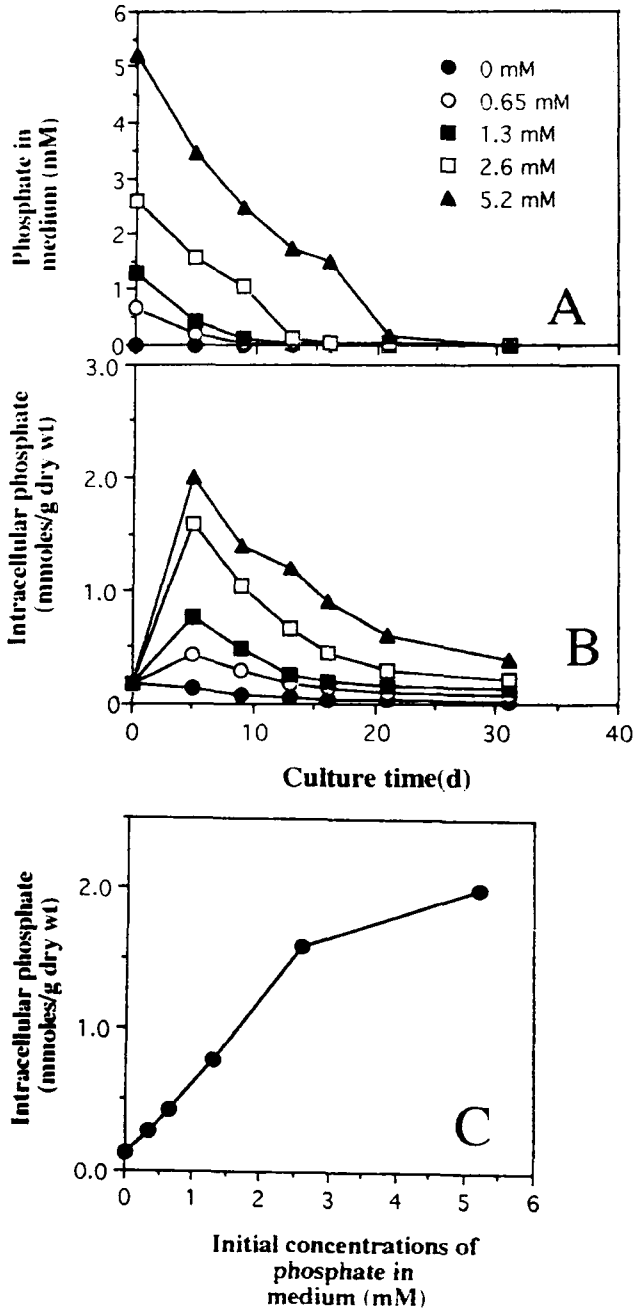

Figure 2. Profiles of medium phosphate and intracellular phosphate of hairy roots cultured in medium containing various concentrations of potassium phosphate during culture period (A, B) and after 5 days of culture (C).

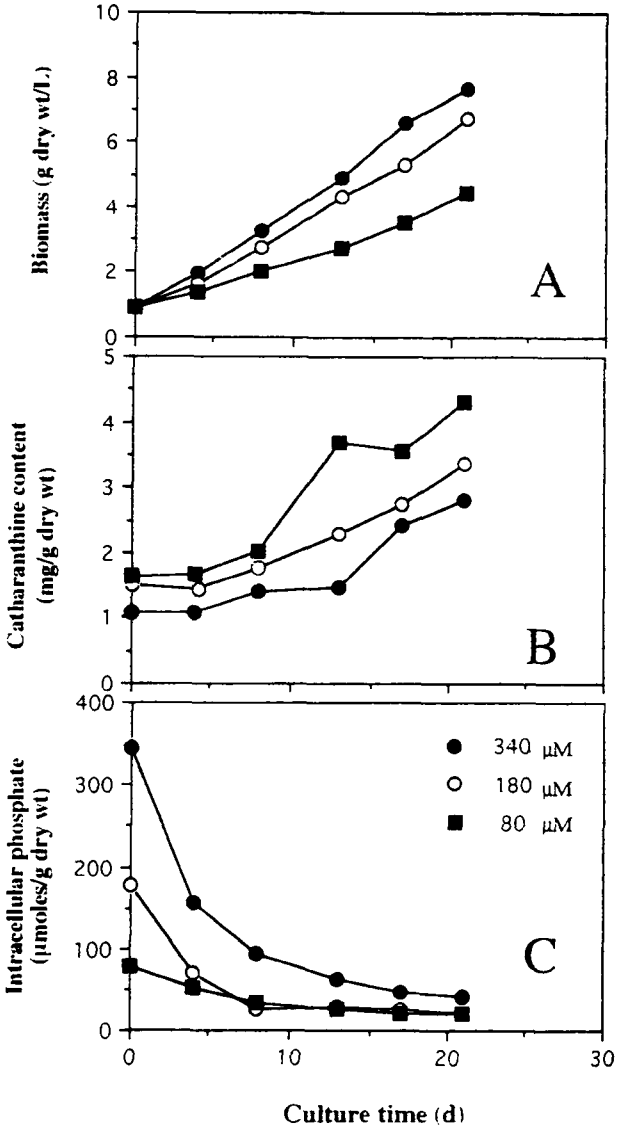

Figure 3. Profiles of growth, catharanthine content, and intracellular phosphate of hairy roots with elevated concentrations of intracellular phosphate during culture period.

The phosphate concentration in the growth medium is shown in Figure 2A. In the case of hairy root cultures with initial phosphate concentrations below 1.3 mM, external phosphate was almost depleted before the exponential culture phase, suggesting that the growth of hairy roots in the exponential phase is maintained by consuming internal phosphate taken up from the medium. In *C. roseus* cell cultures phosphate in the medium is almost entirely incorporated into the cells in spite of a concentration gradient of more than three orders of magnitude (Knolbloch and Berlin, 1983). Thus, the intracellular phosphate level should be investigated to determine whether the phosphate level regulates cell growth and indole alkaloid production. Intracellular phosphate levels of hairy roots in a phosphate-free medium decreased steadily as the hairy roots grew (Fig. 2B). The concentration of intracellular phosphate abruptly increased just before the exponential phase of growth (after 5 days of culture). At the time the intracellular phosphate level was linearly proportional to the phosphate level in the medium (Fig. 2C) after which the concentration of

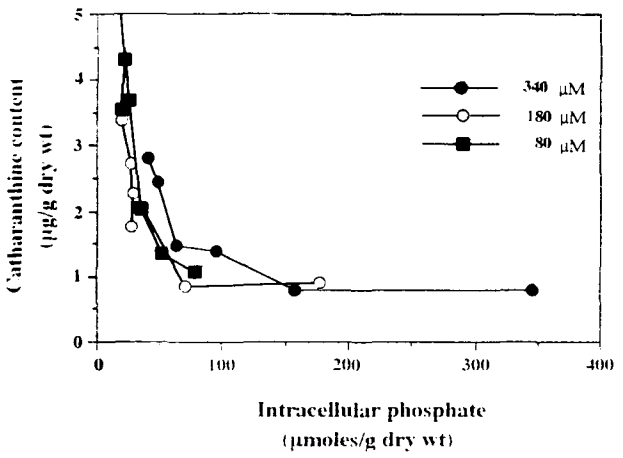

Figure 4. Relationship between the intracellular phosphate level and catharanthine content.

intracellular phosphate decreased as the hairy roots grew vigorously. The effects of phosphate on growth and catharanthine production of hairy roots indicate that limitation of the external phosphate concentration causes the use of the internal phosphate pool, which in turn stimulates indole alkaloid production.

When hairy roots were cultured at various phosphate concentrations, subsequent phosphate uptake and consumption followed. To elucidate the effects of intracellular phosphate on growth and indole alkaloid production, hairy roots with elevated intracellular phosphate levels were cultured in a phosphate-free medium. The growth rate and final biomass of hairy roots increased at the intracellular phosphate level was increased to 80, 180, and 340 μM/g dry wt (Fig. 3A), whereas catharanthine production was strongly stimulated when the intracellular phosphate level was low (80 μM/g dry wt) (Fig. 3B). Since there was no phosphate in the growth medium, the intracellular phosphate level decreased steadily according to the growth of hairy roots (Fig. 3C). The catharanthine content of hairy roots was plotted against the intracellular phosphate level (Fig.4). When the intracellular phosphate level decreased below 100 μM/g dry wt, the catharanthine content abruptly increased.

These results suggest that the intracellular phosphate level regulates growth and secondary metabolite production in *C. roseus* hairy root cultures. Several metabolic pathways appear to be regulated by phosphate, and the formation of secondary metabolites is induced by a limited phosphate supply in the growth medium, the effect of which is reversed by addition of an excess amount of phosphate to the medium (Knobloch and Berlin, 1980). Transfer of *C. roseus* cells to a phosphate-free "induction medium" causes a rapid increase of alkaloid production, which is preceded by an increase of tryptophan decarboxylase activity, a possible key enzyme of indole alkaloid biosynthesis which produces tryptamine from tryptophan and secologanine. The addition of phosphate to the induction medium decreases the accumulation of alkaloids and the direct enzymatic product tryptamine, although a high level of tryptophan decarboxylase activity is retained (Knobloch and Berlin, 1983). Cells cultured in a medium containing low concentration of phosphate may be channelled preferentially to synthesize secondary metabolite precursors, such as tryptamine. The activity of key enzymes in diverting the flow of amino acids from primary metabolism to secondary metabolism is elevated under a limited phosphate supply condition.

In this study changes in the intracellular phosphate level and the catharanthine content of hairy roots were investigated. The growth rate of hairy roots increased in proportion to the medium phosphate concentration. When hairy roots were cultured in a phosphate-free medium, the catharanthine content increased to its highest level. An increased concentration of phosphate caused the catharanthine content to decrease proportionally. Hairy root cultures with elevated intracellular phosphate levels suggest that the intracellular phosphate level plays a key role in the regulation of growth and secondary metabolite production, in which catharanthine production is strongly stimulated when the intracellular phosphate level is less than 100 μM/g dry wt. To increase both growth and catharanthine production in hairy roots, the intracellular phosphate level was maintained below 100 μM/g dry wt by intermittent feeding of phosphate. Even though the intracellular phosphate level was kept low and the growth of hairy roots was enhanced, the catharanthine content did not increase to the level achieved in a phosphate-free medium. Antagonistic regulation of primary and secondary metabolism by the intracellular phosphate level needs further investigation.

적 요

일일초 모상근배양에서 세포내 인산농도와 catharanthine 생산과의 관계를 규명하였다. 모상근의 생장은 생장배지의 인산농도에 비례하여 증가하였다. Catharanthine 함량은 인산을 첨가하지 않은 배지에서 모상근을 배양하였을 때 최대값을 나타내었고 배지내의 인산농도를 증가함에 따라 비례적으로 감소하였다. 그리고 세포내의 인산농도에 비례하

여 모상근의 생장은 증가하나 catharanthine의 함량은 감소하였다. Catharanthine 함량은 g 건조세포당 인산농도가 100 mole 이하일 때에 현저하게 증가하였다. 이상의 결과로 세포내 인산농도는 모상근의 생장과 이차대사산물의 조절에 중요하게 관여함을 시사한다.

ACKNOWLEDGEMENTS - This work was supported by a grant (N81220) from the Korea Ministry of Science and Technology to J.R.L. We thank Dr. Weon-Taek Seo for his critical reading of this manuscript, and Miss Chang Sook Kim for her assistance in manuscript preparation.

REFERENCES

Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorus. Ana Chem **28**: 1756-1758

Ganapathi B, Kargi F (1990) Recent advances in indole alkaloid production by *C. roseus*. J Exp Bot **41**: 259-267

Jung KH, Kwak SS, Kim SW, Choi CY, Lee H, Liu JR (1992) Improvement of the catharanthine productivity in hairy root cultures of *Catharanthus roseus* by using monosaccharides as a carbon source. Biotechnol Lett **14**: 695-700

Jung KH, Kwak SS, Choi CY, Liu JR (1994) Development of two stage culture process by optimization of inorganic salts for improving catharanthine production in hairy root cultures of *Catharanthus roseus*. J Ferment Bioeng **77**: 57-61

Knobloch KH and Berlin J (1980) Influence of medium composition on the formation of secondary compounds in cell suspension cultures of *C. roseus* (L.) G. Don. Z Naturforch **35**: 551-556

Knobloch KH, Berlin J (1983) Influence of phosphate on the formation of the indole alkaloids aand phenolic compounds in cell suspension cultures of *Catharanthus roseus*: comparison of enzyme activities and product accumulation. Plant Cell Tissue Organ Culture **2**: 333-340

Schenk RV, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledones cell cultures. Can J Bot **50**: 197-204

Toivonen L, Ojala M, Kauppinen V (1991) Studies on the optimization of growth and indole alkaloid production by hairy root cultures of C. *roseus*. Biotechnol Bioeng **37**: 673-680

Zenk MH, El-Shagi H, Arens H, Stockigt J, Weiler EW, Deus B (1977) Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. *In* W Barz, E Reinhard, MH Zenk, eds, Plant Tissue Culture and Its Biotechnological Application, Springer-Verlag, Berlin, pp 27-44

(1995년 4월 1일 접수)