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Effect of Nitrogen, Phosphate and Cell Immobilization on Taxol Production from Cell Cultures of *Taxus cuspidata*

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Abstract : The effects of nitrogen, phosphate in modified B5 medium and cell immobilization on cell growth and taxol production were investigated using cell cultures of *Taxus cuspidata*. The ratio of nitrate to ammonium was found to be an important parameter. The ratio of 1 increased taxol production 10-fold, compared to the original ratio of 20 in modified B5 medium. Reducing phosphate concentration inhibited cell growth, but increased taxol production noticeably. Immobilized cells produced a taxol concentration of ~120 g/*l*(Received July 12, 1995; accepted April 30, 1995)

Introduction

Taxol, a diterpene amide originally isolated from the bark of the yew tree, has been recently approved for the treatment of ovarian cancer and breast cancer.[1-3] It is also presently in clinical trials for use in the treatment of other cancers.[4] Despite the increasing demand, the supply of taxol relies on the bark of slow growing trees. Total chemical synthesis of the taxol is reported, but mass production by this method is still difficult due to the complex chemical structure of the compound.[5,6] Thus, cell suspension culture of *Taxus spp.* can be a viable alternative for the production of taxol.

Some efforts have been made to produce taxol in callus or cell suspension culture of *T. brevifolia* or *T. cuspidata* and the effects of medium and culture conditions on cell growth and taxol formation have been investigated.[7-10] However, little information is available on the effect of medium composition and cell immobilization on taxol formation in cell cultures of *Taxus cuspidata*. Therefore, it appears that these parameters are needed to be studied to optimize taxol production from *in vitro* culture of *Taxus spp.* In this study, the effects of concentrations of nitrate, ammonium and phosphate, and cell immobilization on cell growth and taxol production were investigated in cell cultures of *Taxus cuspidata*.

Materials and Methods

Plant material

Callus was induced from *T. cuspidata* trees growing at Suwon, Korea. Explants of young stem were cutted into about 1 cm long, surface-sterilized and rinsed with sterile distilled water. The stem segments were plated onto modified B5 medium containing 5 mg 1-naphthylacetic acid (NAA), 2 g casein hydrolysate, 15 g polyvinylpolypyrrolidone, 30 g sucrose, 7.2 g agar per liter. After cultivations under darkness at 26℃ for 2~3 weeks, actively growing calli were collected and sieved with 80~100 mesh. Suspension cultures were established from collected calli and maintained at 100 rpm in 250 m*l* shake flasks containing 50 m*l* of the same medium above except that it contained 2 mg NAA and 0 g agar per liter. The cells were subcultured under darkness at 26℃ every 3 weeks.

Cell immobilization

Sodium alginate was used for immobilization of cells.[11] The cells at an exponential phase were mixed with 50 m*l* of 2.5% sodium-alginate solution. This mixture was added dropwise with a peristaltic pump in modified B5 medium supplemented with 20 mM of $CaCl_2$. After 30 min in this solution, the beads were rinsed 3 times with modified B5 medium and transferred to 50 m*l* of modified B5 medium.

Culture medium and incubation conditions

For cell maintenance, modified B5 medium containing 2 mg NAA, 2 g casein hydrolysate, 15 g polyvinylpolypyrrolidone and 30 g sucrose per liter was used. For suspended and immobilized cell cultures, the medium described above was modified appropriately. For analysis of nitrogen effects, each of the following formulations was added to the modified B5 medium without potassium

Key words : *Taxus cuspidata*, taxol, phosphate, nitrate, immobilization
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nitrate and ammonium sulfate: potassium nitrate concentrations (3.125, 6.25, 12.5, 25, 50 mM), ammonium sulfate concentration (0.3, 0.6, 1.2, 2.4 mM), or ratio of nitrate to ammonium (1, 5, 10, 20, 30) at a constant nitrogen level of 26.2 mM. For analysis of phosphate effects, the phosphate was added at concentrations of 0, 0.25, 0.5, 1, 2 mM to the modified B5 medium without sodium phosphate. Levels of phosphate were adjusted by adding potassium dihydrogen phosphate and initial sodium ion content was kept at the same level with 2 mM sodium chloride. For analysis of immobilization effects, the medium was modified B5 medium described above except that it contained 0.15 g or 2.9 g $CaCl_2$ per liter. Cell cultures were maintained in 250 m*l* Erlenmeyer flask with 50 m*l* medium at 100 rpm and under darkness at 26℃ for 30 days.

Analytical methods The cell and medium were separated by centrifugation at 1100 g for 20 min. The percentage of cell volume after centrifugation was considered as the packed cell volume (% PCV). A growth index (GI) was calculated as[12]

$$\frac{\text{Final PCV-initial PCV}}{\text{Initial PCV}\times\text{culture time (day)}}$$

The cells recovered from Ca-alginate beads were dissolved in 10 % (W/V) sodium triphosphate. Then cells were centrifuged, and washed twice with distilled water, and dried overnight at 80℃ for dry cell weight (DCW) determination.

The culture broth was extracted with supelclean LC-18 (Supelco, USA). Taxol content was determined by HPLC (TOSOH, Japan) with a UV/VIS detecter and Cu-rosil-B column (Phenomenex, USA). The mobile phase for isocratic elution constisted of 10 mM ammonium acetate and acetonitrile (55 : 45 by volume). Flow rate was 0.9 m*l*/min and measuring UV wavelength was 228 nm. Taxol identification was done by retention time at ~15 min with an authentic standard (Sigma, USA).

Results and Discussion

Effect of Nitrogen

There are two inorganic nitrogen sources such as potassium nitrate and ammonium sulfate in modified B5 medium. Several runs were made to examine the sole effect of potassium nitrate or ammonium sulfate on cell growth and taxol production. When nitrate (in potassium nitrate) was used as the only nitrogen source, cell growth was relatively high in all cases. Cell growth index reached a maximum of 0.032 day^{-1} at 12.5 mM (Fig. 1). In contrast, taxol production was below 20 μg/*l* in all runs. This result agrees with the contradictory relationship between cell growth and secondary metabolite accumulation in plant cell culture.[13]

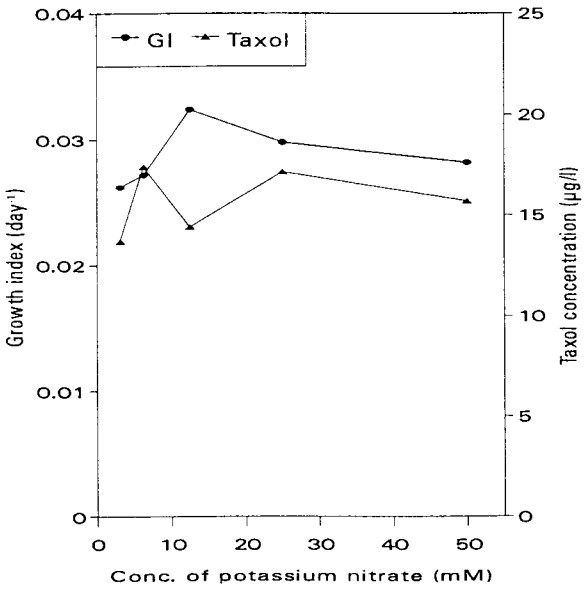

Fig. 1. Effect of nitrate concentration on cell growth and taxol production. Initial cell concentration, 13% PCV; culture periods, 30 days.

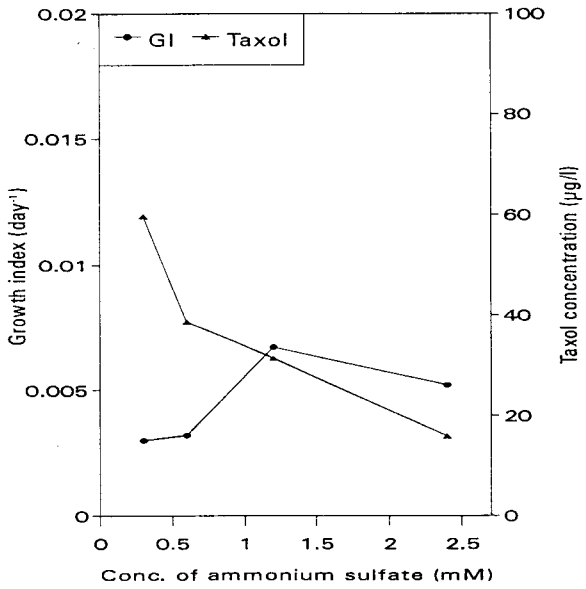

Fig. 2. Effect of ammonium concentration on cell growth and taxol production. Initial cell concentration, 11% PCV; culture periods, 30 days.

When ammonium sulfate was used as the sole nitrogen source, the cell growth was inhibited (Fig. 2), but taxol production was better, compared to potassium nitrate (Fig. 1). The maximun taxol production was 60 μg/*l* at 0.3 mM of ammonium sulfate. The reduced growth

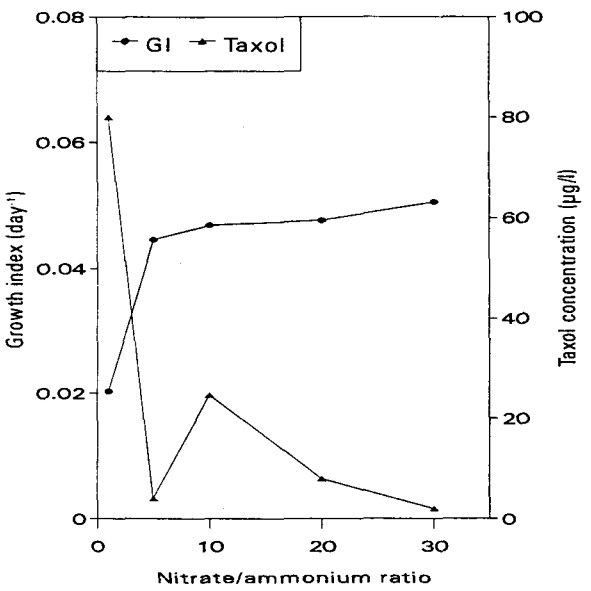

Fig. 3. Effect of nitrate/ammonium ratio on cell growth and taxol production. Initial cell concentration, 10% PCV; culture periods, 30 days.

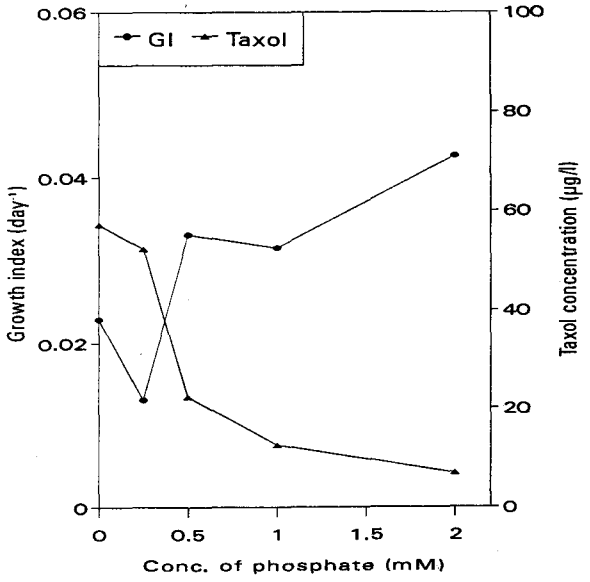

Fig. 4. Effect of phosphate concentration on cell growth and taxol production. Initial cell concentration, 12% PCV; culture periods, 30 days.

of *T. cuspidata* cells at higher concentrations of ammonium sulfate is presumed due to pH reduction and toxicity of ammonium.

When both potasium nitrate and ammonium sulfate was used, the ratio of nitrate to ammonium at a constant level of total nitrogen (26.2 mM) had a significant effect on cell growth and taxol production. As shown in Fig. 3, increasing the ratio of nitrate to ammonium from 1 to 30 was favorable for cell growth, but had an adverse effect on taxol production. The taxol production of 80 μg/*l* was the best at the ratio of 1. It should be noticed that the ratio of 1 increased taxol production 10-fold, compared to the ratio of 20, a value corresponding to the original ratio in modified B5 medium. Since nitrate is a more oxidized form of nitrogen source, it appears that the redox form of the nitrogen source in culture media may play an important role, but this has to be studied further.

Effect of Phosphate

The effect of initial phosphate levels on cell growth and taxol production was studied at phosphate level of 0~2 mM. Shown in Fig. 4, the cell growth was the best at 2 mM of phosphate. The cell growth increased in proportion to the phosphate concentration; however, the taxol content markedly decreased. It is interesting to note that taxol production was the best at 0 mM phosphate. This result indicates that phosphate-limited cells are more metabolically active for the production of taxol. This is consistent with earlier finding on secondary metabolites in other plant cell lines.[14,15] It is presumed that phosphate-limited cells are not effective in primary metabolism, but expose nutritional stress to activate secondary metabolism like taxol formation.

Effect of Immobilization

Immobilization of plant cells are believed to provide the conditions that promote cell differentiation and also increase production of high yields of secondary metabolites.[16] As shown in Table 1, immobilized cells produced a taxol concentration of ~120 μg/*l*. This value was 3.3 times higher than that of suspension culture at the same level of $CaCl_2$. This result is in agreement with that of immobilized *T. cuspidata* cells in glass fiber mats.[7] Our result shows that improvement in product yield was solely due to immobilization effect rather than due to $CaCl_2$ effect as suggested by Humprey *et al.*[17]

It should be pointed out that maximum taxol concent-

Table 1. Effect of cell immobilization on cell growth and taxol production.

Treatment	Initial DCW (g/flask)	Final DCW (g/flask)	Taxol conc. (μg/*l*)	Volumetricproductivity (μg/*l*·day)
Free cells with 0.001 M $CaCl_2$	0.43	0.82	29.7	0.99
Free cells with 0.02 M $CaCl_2$	0.46	0.85	36.2	1.21
Immobilized cells	0.44	0.67	119.7	3.99

ration of ~120 μg/*l* obtained from our study is considerably lower than that of 0.15 mg/*l* reported for the shake flask culture.[9] Although it is necessary to investigate further the improvement of cell line and optimization of culture conditions, our preliminary results are somewhat encouraging. We have demonstrated that manipulations of medium composition and cell immobilization can improve cell growth and taxol production in cell cultures of *T. cuspidata*.

Acknowledgement

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주목 (*Taxus cuspidata*) 세포배양에서 질소원, 인산, 세포고정화가 Taxol 생산에 미치는 영향

박종화, 정인식* (경희대학교 유전공학 연구소 및 유전공학과)

초록 : 주목 (*Taxus cuspidata*) 세포배양을 이용하여 modified B5 배지중의 질소원, 인산 그리고 세포 고정화가 세포생장과 taxol 생산에 미치는 영향을 살펴보았다. Nitrate와 ammonium의 비는 세포생장 및 taxol 생산에 있어 중요한 인자임을 알수 있었고 비값이 1인 경우는 modified B5 배지 조성중 원래 비값인 20에 비해 taxol 생산을 10배나 증가시켰다. 인산의 농도를 감소시킨 경우 세포생장은 저하되었지만 taxol 생산은 현저하게 증가하였다. 고정화세포는 ~120 g/l의 taxol을 생산하였다.

*연락저자