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Effects of Plant Growth Regulators on Growth and Berberine Production in Cell Suspension Cultures of *Thalictrum rugosum*

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Thalictrum rugosum 세포배양에서 식물생장 조절물질이 세포증식 및 Berberine 생산에 미치는 영향

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The effects of various plant growth regulators, both auxins and cytokinins, on cell growth and berberine production were investigated in cell suspension cultures of *Thalictrum rugosum*. Indole-3-acetic acid (IAA) was found to be the best for berberine production among five examined plant growth regulators and the optimum concentration of IAA was 1 μM. The enhancement compared to control 2, 4-dichlorophenoxyacetic acid (2,4-D) was more than 60%. Simultaneous addition of cytokinins such as kinetin and 6-benzylaminopurine (BA) was inhibitory.

A diverse range of important products including pharmaceuticals, flavors and fragrances, insecticides, and other fine chemicals are produced by higher plants (1-3). There remains a continuing interest in the use of plant cell suspension as an alternative source to field cultivation for the production of plant-derived chemicals (4-6). In an ideal situation, this would provide for the continuous, controllable, and reliable manufacture of industrially important chemicals from higher plants in any location.

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 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.

Plant growth regulators, one of the important components of medium, are effective triggers of secondary metabolism. Both the quality and quantity of auxins initially present in media or administered during the course of culture development have a marked effect on primary and secondary metabolism. Plant cell cultures require the addition of growth regulators, auxins and cytokinins, to media for consistent growth by cell division. The effect of auxin type on secondary product synthesis has been investigated in cultures of numerous species. Generally, 2,4-D has been found to be less suitable for triggering secondary metabolism in plant cell cultures than either IAA or 1-naphthalene acetic acid (NAA) (7).

Berberine is a quaternary isoquinoline alkaloid found in several plant species. It has been used as an antibacterial, antiinflammatory, antimalarial and a stomach drug in the Orient. Furthermore, this alkaloid has been shown to posses antineoplastic properties, ex-

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erting its action by intercalating into DNA (8). In addition to its clinical use, berberine is also used as a fluorescent marker in several areas of medical research. Its synthetic derivative, dihydroberberine, is promising as a product against brain tumor since it can penetrate into the blood-brain barrier to accumulate in the oxidized form, berberine, in the brain. Many attempts have been made to produce berberine in callus or cell suspension cultures (9,10), mostly in Japan, and recently it is reported to be under research for commercial production (11).

In this study, effects of plant growth regulators on cell growth and berberine production were examined to get one of the basic informations for the purpose of optimizing berberine production in cell suspension culture of *Thalictrum rugosum*.

Materials and Methods

Plant cell cultures and culture medium

The cell suspension culture of *Thalictrum rugosum* was kindly provided by Dr. Peter Brodelius (Institute für Biotechnologie, ETH, Zürich, Switzerland) and has been maintained on Murashige and Skoog (MS) medium prepared from MS salt mixture (GIBCO) with the additon of 2 μM of 2,4-D, vitamin stock solution and 30 g/*l* of sucrose as carbon source. The pH was adjusted to 6.0 with 1 N KOH. This medium is usually named as MX medium. The suspension cultures were grown in 125 m*l* Erlenmeyer flasks with 50 m*l* of medium on a gyratory shaker (Model G10, New Brunswick Scientific Co.) at 200 rpm. The temperature of the culture room was 25°C and cultures were exposed to 18 hr 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 plant growth regulators 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. When the medium was changed for the replacement of growth regulators, cells were filtered, washed and transferred into new medium. After filteration, 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°C 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 μ *l*) was injected into the HPLC system with a UV detector (Kratos Corp.). 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 and the measuring UV wavelength was 265 nm.

Results and Discussion

There have been numerous reports on hormonal conditions to enhance the yields of secondary product formation, but it is obvious that there is no general rule as usual in most cases of plant cell cultures. Selection of an appropriate combination of growth regulators is often critical to the synthesis of a desirable metabolite even though the precise biochemical mechanism is not well understood (12). Since 2,4-D was the only growth regulator in the MX medium, initial experiments were done in the concentration range of 1 to 10 μM 2,4-D. In most cases of secondary metabolite production, 2,4-D inhibited the product formation even though it supported good growth (13). Therefore, other auxins as well as cytokinins were tested afterward. Fig. 1 displays the effect of 2,4-D concentrations on cell growth and berberine producion. The growth data were normalized against a control cul-

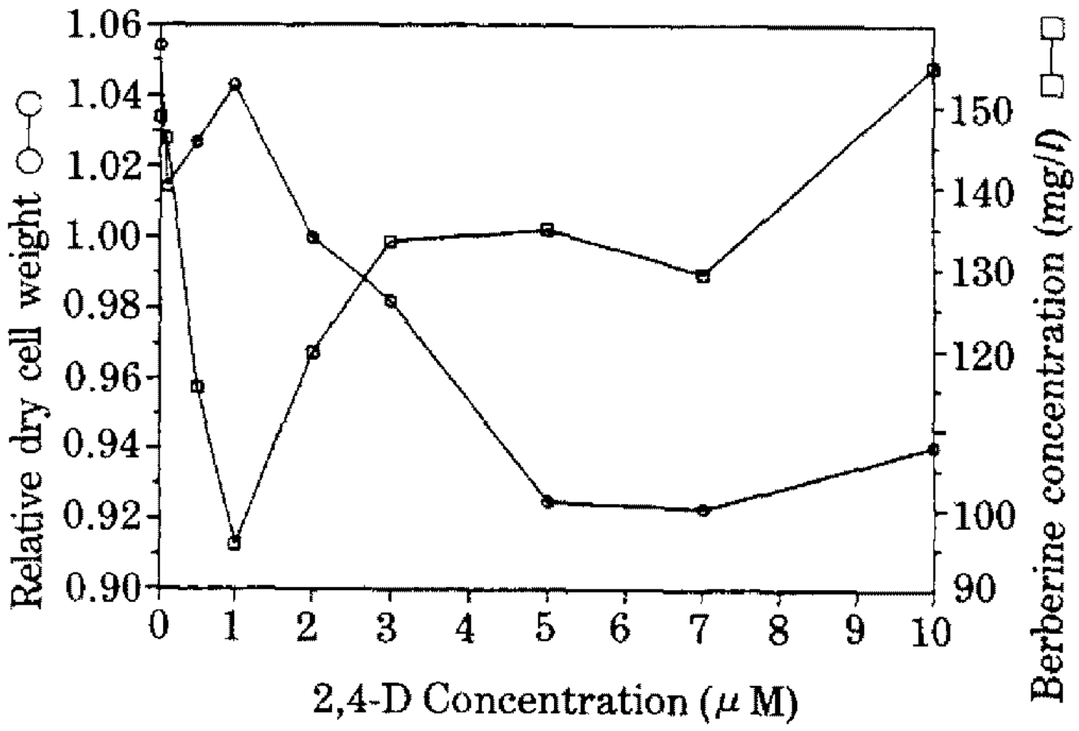

Fig. 1. Effects of 2,4-D on cell growth and berberine production. The growth data were normalized against a control culture with 2 μM of 2,4-D.

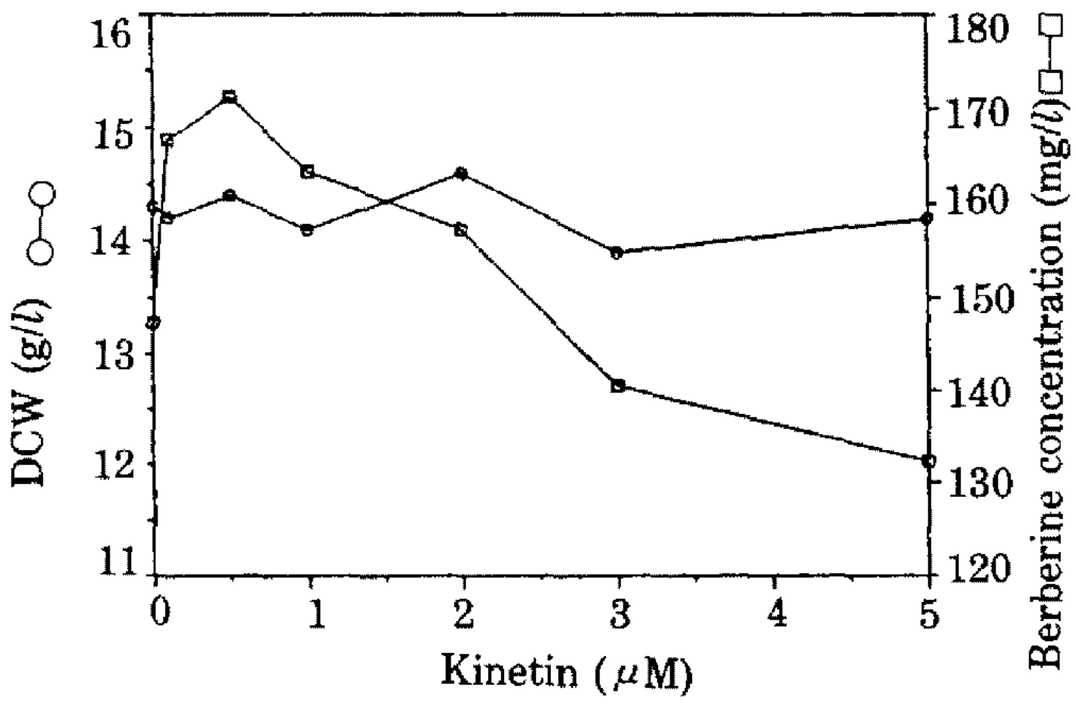

Fig. 2. Effects of kinetin on cell growth and berberine production.

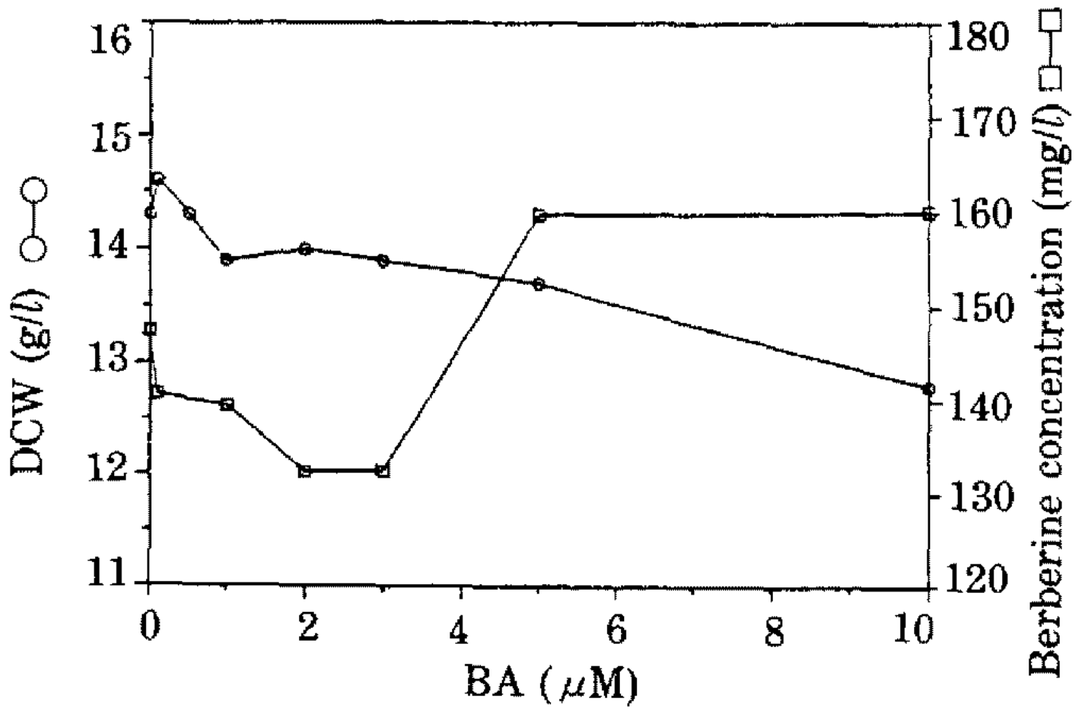

Fig. 3. Effects of BA on cell growth and product formation.

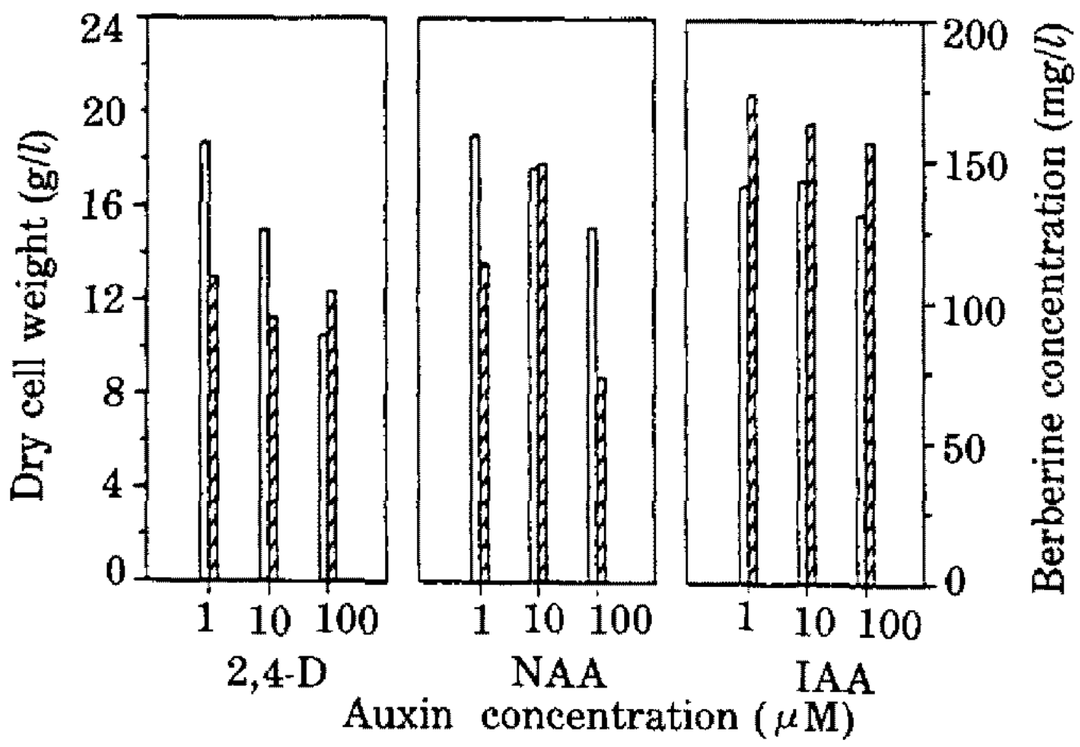

Fig. 4. Influences of three kinds of auxin on cell growth and berberine production. White bar represents dry cell weight and dark bar represents berberine concentration.

ture with 2 μM of 2,4-D. With concentrations higher than 2 μM, berberine production was somewhat enhanced in spite of the decrease in cell mass. At 0.1 μM of 2,4-D or without 2,4-D, both growth and product formation were slightly better than those of control culture. However, long-term subcultures with this low level of 2,4-D may not support cell suspension growth. As shown above, changes in 2,4-D concentration resulted in slight improvement for production, but it was not significant.

The effect of cytokinins, kinetin and 6-benzylamnopurine (BA), was examined in addition to 2,4-D. Fig. 2 and 3 show the influences of kinetin and BA respectively. At low concentration of kinetin (0.1, 0.5, and 1 μM) and at high concentration of BA (5 and 10 μM), berberine level was higher than that of the control (without cytokinins). Kinetin did not affect cell growth much. As the concetration of BA increased, the cell growth decreased slightly.

According to Tabata *et al.*(14), kinetin promoted nicotine production in the absence of auxin, whereas auxin strongly inhibited nicotine formation even in the presence of kinetin, without affecting tissue growth. On the other hand, berberine-producing activity was remarkably enhanced by simultaneous administration of auxin and cytokinin in cell suspension cultures of *Thalictrum minus* (15). The selection of auxin also influenced the product formation considerably. In *Catharanthus roseus* culture, 2,4-D and NAA suppressed alkaloid formation while IAA gave high cell and alkaloid yield (16). Koul *et al.* (17) also reported similar results in suspension cultures of *Hyoscyamus*, in which the natural auxin IAA promoted alkaloid synthesis when compared to synthetic auxin 2,4-D and NAA. To decide the best auxin for berberine production in *T. rugosum* culture, three auxins (2,4-D, NAA,

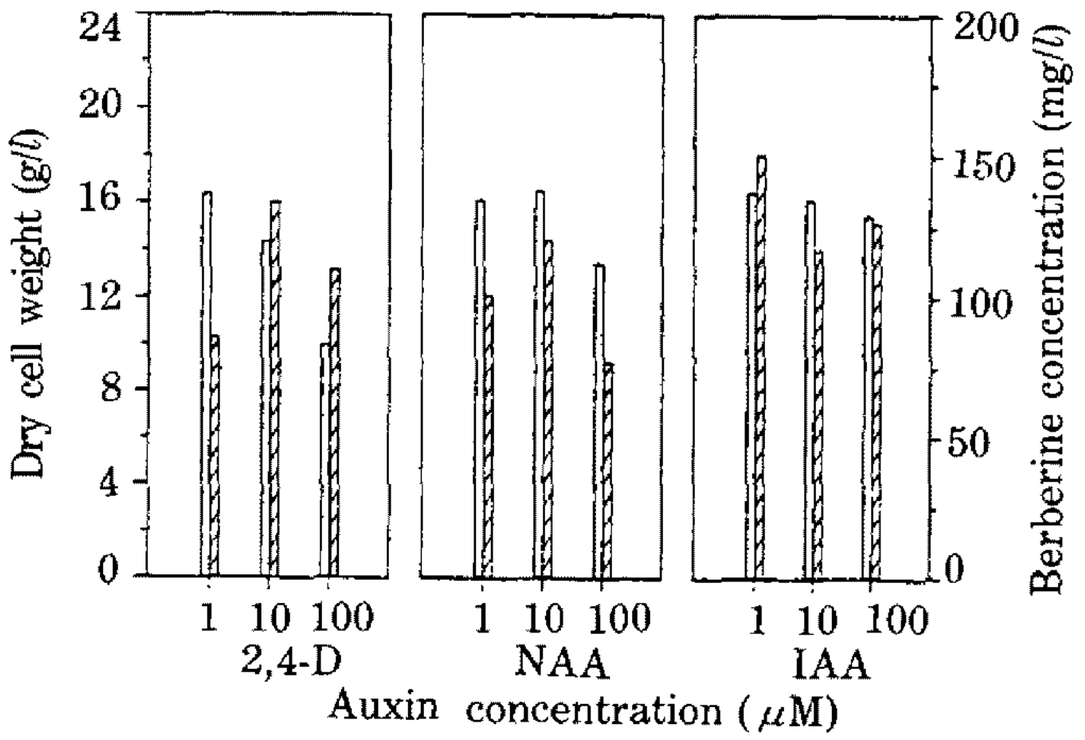

Fig. 5. Effects of three different auxins with simultaneous administration of BA on cell growth and berberine production. White bar represents dry cell weight and dark bar represents berberine concentration.

and IAA) were examined up to 100 μM. Their effects on cell growth and berberine production are depicted in Fig. 4 and the effects of the same auxins administrated with BA are shown in Fig. 5. High concentration of 2,4-D suppressed cell growth severely while product formation was enhanced. This resulted in significant increase of specific productivity. In addition, almost one-half of berberine produced at 100μM of 2,4-D was found in the medium which had to do with the permeability of cells. At 1 and 10 μM of NAA, both cell growth and product formation were stimulated. Optimum concentration of NAA for berberine production was 10 μM at which 37% increase of product yield was observed. At 100 μM of NAA, berberine production was reduced remarkably. The culture with IAA generally enhanced the product formation significantly even though the final cell mass was lower than that of culture with 2,4-D. At 1 μM of IAA, 173,4 mg/*l* of total berberine was produced which was more than a 60% increase. Higher concentration of IAA also supported good productivity, though the levels were a little lower without affecting growth. Therefore, it is evident that IAA is the best auxin for the optimum production of berberine.

In conclusion, IAA was selected as the best auxin for berberine production in cell suspension cultures of *T. rugosum* and the optimum concentration was 1 μM.

요 약

Thalictrum rugosum 세포배양에서 여러 가지 식물생장 조절물질이 세포증식 및 berberine 생산에 미치는 영향을 조사하였다. 조사된 식물생장 조절물질들 중에서 indole-3-acetic acid(IAA)가 berberine 생산에 가장 적합함을 알 수 있었고 그 최적농도는 1 μM이었다. 비교기준인 2,4-D의 사용결과에 비할 때 60% 이상의 생산량 상승효과가 있었으며, cytokinin인 6-benzylaminopurine(BA)를 동시에 사용하는 것보다 IAA만을 단독으로 사용하는 것이 더 효과적이었다.

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