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Berberine Production by Cell Suspension Cultures of Cork Tree (*Phellodendron amurense* Rupr.)

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Abstract – Various culture conditions for cell growth and berberine production in cork tree (*Phellodendron amurense* Rupr.) were investigated. Callus was induced from cambium tissue of cork tree, and cultured on LS liquid medium supplemented with 0.5 mg/l 2,4-D, 0.1 mg/l BA, and 3% sucrose. Several factors enhancing berberine production and cell growth in cork tree cell cultures were found. Some of them enhanced both cell growth and berberine production, but others resulted in a decoupling of cell growth and berberine production with significant in the specific levels. High level of nitrate (80 mM), high level of phosphate (8.98 mM), and sucrose (7%), 1.0 mg/l IAA were effective in berberine production, whereas low level of nitrate (40 mM), and phosphate (2.25 mM), and high level of sucrose (7%) in the medium were effective in cell growth. Two stage culture(first stage for cell growth, and second stage for berberine production) increased berberine production almost twice (5.06 mg/g dry weight) as much as single stage cultures in berberine production.

Key words – berberine; two stage cultures; secondary metabolites.

Plant secondary metabolites have been known to play a major role in the quality of food, favour and dye, and insecticide[1]. Plant cell cultures could be an alternative approach to the production of such secondary metabolites. Although in the past years all economically important plants have been brought into cell culture, however, in most cases the productivity was too low to allow an economically feasible process.[2] Selection of stable cell line and finding of optimum conditions for cell growth can solve these problems. Optimal conditions for the increase of both cell growth and product yield can be found by changing the chemical and physical factors in culture. Chemical factors are medium components such as nitrate, phosphate and phytohormones. Aeration, temperature, pH in the culture media, and light belong to physical factors.[3]

Cell growth and metabolite production are generally shown to be negative correlation. Thus, decoupling of cell growth and product formation stages by use of two stage batch or continuous culture is necessary for optimal biosynthesis of a non-growth associated products.[4]

Berberine has been used as a fluorescent marker in several areas of medicinal research as well as antibacterial, antimalarial and stomach drug in the Orient.

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In this study, various conditions for two stage cultures were established in order to enhance berberine production and cell growth of *Phellodendron amurense* Rupr.

EXPERIMENTAL

Suspension culture – Suspension culture of cork tree (*Phellodendron amurense* Rupr.) as initiated by transferring callus to liquid MS[13] medium supplemented with 3% sucrose, 1.0 mg/l 2,4-dichlorophenoxy acid (2,4-D), and 0.1 mg/l benzylamino purine (BA). Cultures were placed in the same medium in the dark condition at 25°C and were subcultured every 4 weeks. The effect of various macronutrients was tested by transferring duplicate samples of 1.5 g callus of 10-day old suspension into 250 ml Erlenmeyer flask containing 50 ml of test medium. The test media for studying the effect of nitrate, phosphate, sucrose, and growth regulators (2,4-D, IAA and BAP) were prepared by modifying their concentrations in the LS[14] basal medium. All cultures were maintained in the dark condition at 25°C on a gyratory shaker.

Two stage culture – Based on the results obtained in suspension culture, growth medium (GM) and production medium (PM) were formulated for two stage cultures. Twenty-five days after culture in growth medium, medium was replaced with fresh production medium. Cells were harvested by suction filtration after 10-day incubation period in the test medium. Dry weight was measured after desiccation at 60°C for 24 hr.

Determination of berberine – Samples for berberine analysis were collected by taking 0.5 g of cells (fresh weight) and analyzed using the described method by Nakagawa.[15] Dried cells were then extracted with 10 ml methanol in a sonicating bath at 50°C for 1 hour. Quantitative determinations of berberine and palmatine were made by the calibration curve obtained with authentic compounds using a Spectra Physics SP8800 HPLC system equipped with a UV detector. Separation was accomplished using μ-Bondapak C18 (300×3.9 mm) column: solvent 1 mM tetrabutylammonium phosphate (pH 2.0) : acetonitrile (40 : 60); flow rate 0.5 ml/min; wavelength 265 nm. Berberine was tentatively identified based on retention time and UV spectra.

RESULTS AND DISCUSSION

Suspension cultures – Primary callus cultures were established from stem cambial tissues of *Phellodendron amurense* which cultured on LS liquid medium with 0.5 mg/l 2,4-D, 0.1 mg/l BA, and 3% sucrose. Callus tissues rapidly grew on LS liquid medium with 0.5 mg/l 2,4-D, 0.1 mg/l BA, and 3% sucrose. However, initially the cell suspension cultures may be quite different in appearance and growth.

Berberine was detected in extract of cultured cells, but the amounts also varied depends on cell lines and culture conditions. HPLC chromatograms of berberine and palmatine are shown in Fig. 1.

The effect of nitrogen source on both cell growth and berberine production was examined by varying concentration of NH_4NO_3 and KNO_3 (Fig. 2). High nitrate concentration in the medium resulted in a marked increase in berberine production,

Fig. 1. HPLC separation of authentic berberine compounds and extracts of suspension cultured cells of *Phellodendron amurense* Rupr. A: Authentic berberine and palmatine, B: Cell extracts, C: Co-chromatography of authentic compounds and cell extracts(A+B)

Fig. 2. Effect of nitrate concentration on cell growth and berberine production.

Fig. 3. Effects of phosphate concentration on cell growth and berberine production.

Fig. 4. Effects of surcrose concentration on cell growth and berberine production.

whereas low level nitrate promoted cell growth. For examples, the high berberine production was obtained in the medium containing 80 mM nitrate, while cell growth was to be shown 40 mM. Nitrate has been known to play an important role in the production of secondary metabolites[3]. It was also reported that high nitrate concentration suppresses cell growth of hybrid poplar, but promotes anthocyanin production.[5]

Cell growth was prompted by phosphate in the medium up to the concentration of 54.8 mM. Higher phosphate level than this did not promote cell growth. However, cell growth was promoted exceptionally at the level of 2.25 mM phosphate (Fig. 3). The optimal concentration for berberine production was 8.98 mM. This optimal initial phosphate concentrations is half times of that in normal LS medium. The internal phosphate level seemingly played a key role in the cell growth and secondary metabolism.[3]

The effect of sucrose on cell growth and berberine yield was investigated. Cell growth as well as berberine yield increased with sucrose concentration (Fig. 4). The optimal concentration of sucrose for both berberine production and cell growth was 7%. Sucrose has been known

Table I. Effect of growth regulators on cell growth and berberine production

Growth regulators (mg/l)			Cell growth (g dry wt/ flask)	Berberine content (mg/g dry wt)
2,4-D	IAA	BA		
1.0	1.0	-	0.098	0.056
1.0	5.0	-	0.116	0.340
1.0	10.0	-	0.074	0.254
-	1.0	-	0.075	3.170
-	5.0	-	0.091	0.051
-	10.0	-	0.120	0.750
-	5.0	0.5	0.103	0.056
-	10.0	0.5	0.398	0.056
5.0	-	0.5	0.090	0.690

to support high growth rate and biomass yield in plant cell cultures.[3]

In cork tree cells, cell growth and berberine production needed different growth regulators. 2,4-D and IAA promoted growth of cells (Table I). Although cell growth in medium with high concentrations of 2,4-D and IAA markedly superior to low concentrations and non-treatments, berberine production was inhibited by high level of auxin. Addition of 2,4-D has been known to be responsible for suppressing formation of secondary metabolites in many plant cell culture systems.[6,7]

Berberine production in cork tree cells was stimulated when IAA was incorporated with cytokinin (BA). This observations suggested important role of BAP as a promoter of berberine production. Hara *et al*[9] reported that BA activated enzymatic reactions subsequent to the formation of the amines in the biosynthesis of berberine. The highest berberine yield was obtained from LS basal medium containing 1.0 mg/l IAA.

Cell suspension cultures of *Acer pseudoplatanus*,[10] *Catharanthus roseous*,[11] tabacco,[12] have shown that accumulation of secondary metabolites is very sensitive to nutrient supply.

Table II. Comparison between one stage culture and two stage culture on cell growth and berberine production

Cultures	Cell growth D.W. (mg/flask)	Berberine content (mg/g dry wt)
One stage cultures (GM→GM)	250±30*	2.350±359
Two stage cultures (GM→PM)	290±45	5.065±698

*The values represent the mean±standard deviation.

Two stage cultures - When the optimal condition for cell growth is different from that for synthesis of secondary metabolites, a two stage culture system can improve productivity by optimizing conditions separately for cell growth and product formation. Growth medium and production medium were formulated based on the previous results for optimal nutrient constituents. It was noteworthy that several conditions such as changing concentrations of nitrate, phosphate, carbon source, and growth regulators. Thus, we attempted to find conditions for two stage cultures to ensure both maximum growth in GM (growth medium) and maximum production in PM (production medium). Ten-days after two stage culture, the berberine content in cork tree cells was increased to 5.06 mg/g dry weight (Table II). This result was different to that of a previous report.[11] They reported that transfer of *Catharanthus roseous* hairy root from GM to PM reduced the growth rate.

This study showed the optimization of chemical condition and establishment of two stage culture can enhance the product yield of plant secondary metabolites. Further study is being underway to refine this study by modifying salts, growth re-

gulators, and carbon sources in culture medium.

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