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식물세포 배양 및 융합을 통한 유용물질 개발(II)

김길웅* · 박용구* · 최명석*

Development of Useful Products Through Plant Cell Fusion and Culture of *Populus spp.*(II)

Kim. K.U., Y.G. Park and M.S. Choi

ABSTRACT

Anthocyanin formation in callus cultures using *Populus alba* × *Populus glandulosa* was evaluated on basal MS medium supplemented with various levels of growth regulators, sucrose and nitrate concentrations. The highest yield of anthocyanin from cultured cells was produced under 5% sucrose, 1/8 strength of nitrate(12.5% of basic concentration) and combination of 1.0 mg/l IAA with 2 mg/l BAP, respectively. The high anthocyanin producing cell line no. 11 was selected among 15 cell lines, showing over 80% cells contained anthocyanin producing cells. From these cells, the highly productive red protoplast was isolated and the highest protoplast yield, 6.7 × 10^6 was obtained in enzyme combination IV which is composed of 2.0% cellulase, 0.5% macerozyme and 0.1% pectolyase.

Key words : Anthocyanun, *Populus alba* × *Populus glandulosa*, useful products

INTRODUCTION

Plants have for a long time been of great importance not only as food sources but also as a supply of wide range of chemicals including pharmaceuticals, insecticides, flavors, flagrances and colors.[7] There have been many attempts to establish tissue culture method for the commercial production of useful plant metabolite.[4] Among the approximately 2,000 flavonoids, some accumulated in tissue and cell cultures. Anthocyanin also belongs to the flavonoids that occur widely in gymnosperms, monocotyledons and dicotyledons. They are of chemotaxonomic value and also play a role as genetic markers.[5]

Recently, many attempts have been made to improve the productivity of cell cultures by both optimization of environmental conditions and the selection of high producing cell lines.[9] Thus far, only a few investigators have used protoplast culture system for development of secondary metabolites. Recently, selection of high-producing

* 경북대학교 농과대학(Dept. of Agronomy, Kyungpook National Univ., Taegu 702-701, Korea)
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cell line has been reported for shikonin derivatives from protoplast cultures of *Lithospermum erythorihizon* cells. Growth regulators significantly influence cell growth and morphology. High concentration of auxin can lead to improvement in taxol yield from cell culture medium of *Tasus spp.*[2]

However, there are not many reports on the biosynthesis of anthocyanin or mechanism of anthocyanin formation in the woody plants. This study was carried out to investigate the effect of various factors on the anthocyanin formation in order to establish the stable cell lines of *Populus alba* × *P. glandulosa.*

MATERIALS AND METHODS

The medium used for callus initiation was the modified MS medium supplemented with 1.0 mg/l 2,4-D, 0.1 mg/l BAP and 3% sucrose. The various levels of sucrose(1-10%), nitrate(0-400% of control) and plant growth regulators(0-2 mg/l) based on MS medium were tested for their effects on anthocyanin production.

After 13 days from initial culture, cell growth was expressed as gram fresh weight per test tube and anthocyanin content was measured by UV spectrophotometer an 530 nm with 0.5 g fresh weight per test tube.

Preliminarily, anthocyanins in the crude extract from the anthocyanin production callus with methanol containing 1.5% HCl(v/v) were separated by two dimensional thin layer chromatography with the solvents of BAW and AAH. At least five different anthocyanins were analyzed by thin layer chromatography and measurement of absorption spectrum at 530 nm.

Fifteen cell lines, high anthocyanin accumulated cells and low anthocyanin accumulated cells were selected by the following procedures. Cell cluster(ca. 0.5 g fresh weight) with accumulated anthocyanin cell lines was isolated from original callus cultures with the naked eye. Each cell cluster was placed on a 20 ml of MS media cultured at 26+1℃ under fluorescent light of 7,000 lux for 13 days.

After the pigmented cells were macerated with cellulase "Onozuka"R-10 and pectolyase Y-23[11], percentage of anthocyanin synthesizing cells was calculated from the proportion of protoplasts which were visibly red color in cytoplasm under microscope. Protoplasts were isolated by the same methods of Park and Son.[11] The petridishes($1\text{-}3\times10^5$) were sealed with parafilm and cultured under the dim light. After seven days of plating, the frequency of cell division was measured using an inverted microscope. The frequency was expressed as a mean number of dividing protoplasts in petridish.

RESULTS AND DISCUSSION

Cell growth and anthocyanin production were increased as sucrose concentrations increased from 1% to 5%(Fig. 1). The highest anthocyanin production was observed at 5% sucrose medium. It has been known that the best sugar for production of anthocyanin was sucrose in cell cultures of maize[12] and poplars hybrid.[6] Various concentrations of NH_4NO_3 and KNO_3 were used to evaluate their effects on anthocyanin formation or cell growth in *Populus* cells(Fig. 2). High nitrate concentrations limited cell growth and anthocyanin synthesis, but the lower concentration of nitrate such as 1/8 strength of basic concintration(12.5%) increased anthocyanin production, with the most significant cell growth at 1/2 strength of basic nitrate concentration(50%). Dougall[3] reported that nitrate plays an important role not only in cell growth but also in secondary metabolism in higher plant.

Among auxin type growth regulators, 2,4-D

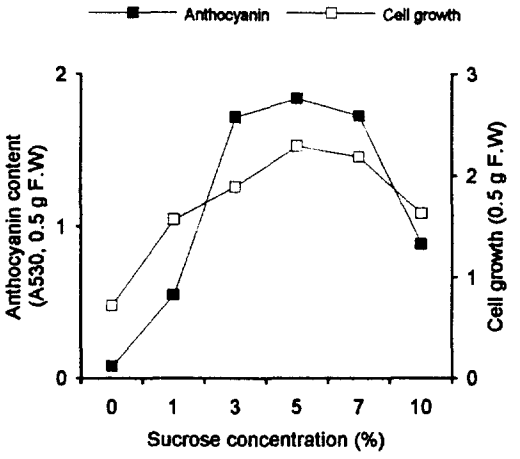

Fig. 1. Effect of sucrose concentration on cell growth and anthocyanin production in callus of *P. alba* × *P. glandulosa*. Cells were cultured on MS agar medium supplemented with 0.5 mg/l 2,4-D, 0.1 mg/l BA under continuous illumination cell growth was measured after 13 days of cultures.

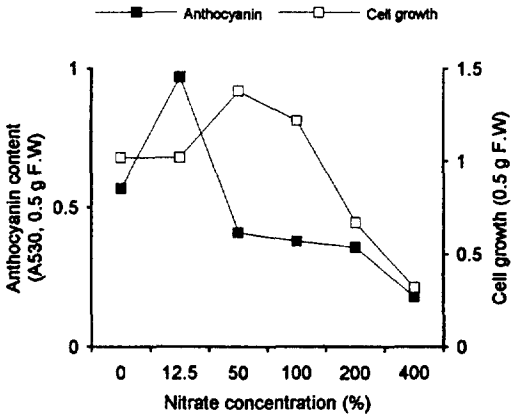

Fig. 2. Effect of nitrate concentration on cell growth and anthocyanin production in a callus of *P. alba* × *P. glandulosa*(the ratio of KNO_3: NH_4NO_3 was 1 : 1)

exerted better effect than others like IAA or NAA in cell growth. However, the high concentrations of 2.4-D markedly inhibited anthocyanin production compared with two others(preliminary study). For anthocyanin production combination of 1.0 mg/l IAA with 1.0 mg/l BAP seemed to

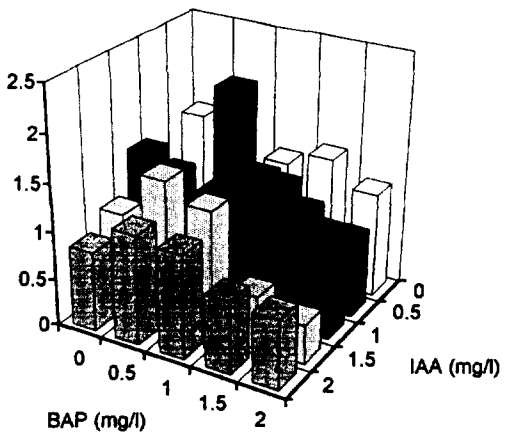

Fig. 3. Effect IAA vs. BAP concentrations on anthocyanin formation of *P. alba* × *P. glandulosa*. Cells were cultured MS agar medium supplemented with 3% sucrose under continuous illumination for 14 days.

be suitable concentrations(Fig. 3). Choi[2] reported that high concentration of auxin, promoted taxol production from cultured cells of *Taxas spp.* This indicates that plant growth regulators have different effects on secondary metabolites depending on plant species.

Anthocyanins were identified from purified extracts by TLC with four solvents system: n-butanol/acetic acid/water(4 : 1 : 5, v/v/v), n-butanol /2N HCl(1 : 1, v/v), aqueous 1% HCl and acetic acid/concentrated HCl/water(15 : 3 : 82, v/v/v/). Phelargonidin 3-rhamnoside was identified from purified extracts, by comparison of their Rf values and spectra of the authentic samples (Table 1).

Among 15 cell lines, cell line 11 produced the hightest anthocyanin, showing more than 80 cells containing producing cells, as compared with cell line 4 contained only 33% of cells producing anthocyanin(data not presented).

Fig. 4 shows the production of anthocyanin in poplar cells by culturing days. The highest yield was obtained at the 6-day-old callus subculture while that of red protoplasts i.e. anthocyanin containing protoplasts increased as the subculturing

Table 1. Rf values and spectral data of purified antocyanin from the *Populus alba* × *Populus glandulosa* callus.

Pigment	Rf values in solvent[1] (Rf × 100)				Color	Max[2]	E440/Emax
	A	B	C	D			
Purified anthocyanin	45	26	37	70	red	507	21
Pelargonidin 3-rhamnoside-5-glucoside	46	24	39	70	red	505	19

[1] Mark Keisel 60F254 was used decending chromatography with the following solvent systems: A : n-butanol/acetic acid/water (4 : 1 : 5, v/v/v), B : n-butanol/2N HCL(1 : 1, v/v), C : aqueous 1% HCl and D : acetic acid/conc. HCl/water(15 :3 : 82, v/v/v).

[2] Spectral data were determined in methanol containing 0.01% HCl.

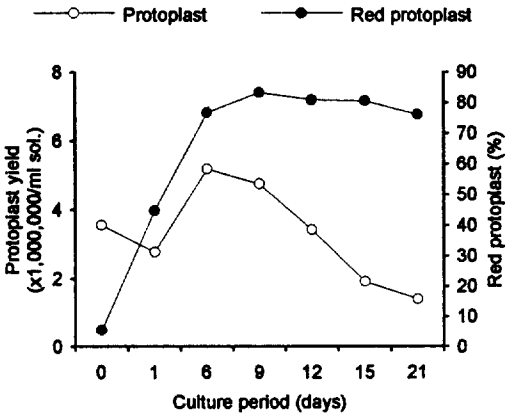

Fig. 4. Effect of culture of callus on the protoplast yield and on the frequency of red protoplasts of *P. alba* × *P. glandulosa*.
Protoplast yield(o—o):
Red protoplasts(●—●)

period increased(Fig. 4). It has been known thatprotoplast yield was markedly influenced by the growth phase of callus.[8] But it was observed at 10-day-old callus subculture in non-pigmented cells of *P. alba* × *P. glandulosa*. These results suggested that protoplast yields were closely related with subculturing period.

The ratio of cell amount and kind of enzyme also influenced protoplast yield and viability. Park and Han[10] reported that the highest protoplast yield of *P. alba* × *P. glandulosa* were observed in enzyme solution mixed with cellulase "Onpzuka" R-10 drisellase, macerozyme R-10, and pectolyase Y-23. The highest yield of protoplast, 6.7×10^6 was obtained in enzyme combination IV, which was composed of 2.0% cellulase, 0.5% macerzyme and 0.1% pectolyase(Table 2). This enzyme combination seems to be the most suitable one to isolate protoplast from callus.

Protoplast of *P. alba* × *P. glandulosa* grew well in MS medium with absence of NH_4NO_3. But in this modified MS medium, cell division was sustained and colony was formed(Table 3).

Table 2. Effect of four enzyme combination on protoplast yield of *Populus. alba* × *Populus glandulosa*.

Types of enzyme	I	II	III	IV
Protoplast yield ($\times 10^6$/gram FW)	4.1	3.6	4.7	6.7

Table 3. Effect of different culture medium on cell division and colony formation of *Populus alba* × *Populus glandulosa*.

Medium types	Cell division	Colony formation
KM-8p	21.1 + 11.8[1]	++[2]
MS	16.1 + 10.5	+
MS-NH_4NO_3	17.5 + 12.6	++
WPM	12.1 + 0.5	+

[1] Each value represents the mean SD of 3 replication experiments.

[2] Visual estimation: + poor, ++ good, +++ very good.

The deleterious effects of ammonium in medium have been reported in the protoplast culture of *Lycopersicon spp.*[13] and potato.[1]

Using this protoplast selection system, it would be possible to obtaine the homogenity cell line and protoclone for high anthocyanin productivity.

Thus, these results can be applied for selection of high productivity cultured cells for secondary metabolite.

摘 要

현사시나무의 조직 배양 세포에서의 anthocyanin 생산성이 높은 세포계를 얻기 위한 목적으로 실험을 수행한 바 다음과 같은 결과를 얻었다.

Anthocyanin 생산력이 높은 세포계의 선발은 sucrose 3%, 2, 4-D 0.5 mg/l, BAP 0.1 mg/l가 첨가된 MS기본배지에서 실시하여 15개 cell line중에 생성력이 80%에 달한 ACL 11 세포계를 선발하였다. Anthocyanin 생성에는 배지 환경요소가 중요한 인자로 작용함을 밝혀 냈다.

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