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Anthocyanin Synthesis in Cell Cultures of *Populus alba* L. × *P. glandulosa* Uyeki[1]*

Young Goo Park[2], Myung Suk Choi and Sung Ho Son

細胞培養을 利用한 현사시나무의 안토시아닌 生成[1]*

박용구[2] · 최명석[2] · 손성호[3]

ABSTRACT

The influence of various levels of major medium components such as sucrose, nitrate, phosphate, plant growth regulators, and light intensity for cell growth and the production of anthocyanin content in cell cultures of *Populus alba* X *P. glandulosa* were investigated. Best results for anthocyanin yield were obtained using Murashige and Skoog(MS) medium containing 5% sucrose, 12.5% nitrate, 200% phosphate, 1.0mg/l indole-3-acetic acid(IAA), 1.0mg/l benzylaminopurine(BAP), and continuous illumination of 7,000 lux. On the other hand, maximum cell growth was achieved with 5% sucrose, 50% nitrate above 400% phosphate (compare with that of MS basal medium), and 0.5mg/l 2,4-dichlorophenoxyacetic acid(2,4-D). Anthocyanin accumulation in a suspension cultured cells of given genotype was stimulated by subculturing onto the medium lacking 2,4-D. Pigmented cell clusters were extracted with methanol containing 1% hydrochloric acid(HCl) and then anthocyanin was identified by thin layer chromatography(TLC) and U.V. spectrophotometer.

Key Words : *Pigmented cells, production medium, secondary metabolites, suspension culture.*

摘 要

기내배양된 현사시나무의 조직배양세포로부터 안토시아닌 생성과 세포생장에 적합한 요인을 구명하여 차후 기내배양을 통한 2차대사산물생산의 기초자료를 제공하고자 연구를 수행하였다.

Callus는 0.5mg/l 2,4-D, 0.1mg/l BAP를 첨가한 MS배지에서 유발시켜 같은 배지에서 증식하였다. 안토시아닌 생산은 MS 기본배지를 기준으로 하여 질산염은 12.5% 감량시키고 인산염은 400%로 증가시켜 5% sucrose와 1.0mg/l IAA 및 1.0mg/l BAP를 첨가한후 7,000 lux의 연속광 하에서 배양했을 때 가장 높게 나타났다. 그러나 세포의 생장은 MS 기본배지를 기준으로 하여 질산염은 50% 감량시키고 인산염은 400%로 증가시켜 5% sucrose와 0.5mg/l 2,4-D를 첨가한 배지에서 가장 양호한 결과를 얻었다. 안토시아닌의 동정은 1% 염산-메탄올의 유기용매로 추출하여 정제 후 TLC와 UV spectrophotometer로 확인한 결과 pelargonidin 3-rhamnoside-5-glucose로 추정되었다.

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[2] 慶北大學校 農科大學 Department of Forestry, Kyungpook National University, Teagu, 702-701, Korea.
[3] 林木育種硏究所 Institute of Forest Genetics, Suwon 441-350, Korea.
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INTRODUCTION

Anthocyanins are widespread group of coloring matters in plants. The water soluble pigments are largely responsible for blue, mauve, violet, and red in flowers, fruits, and/or leaves(Nozue et al., 1987).

Production of useful substances such as alkaloids, steroids, vitamines etc. by plant tissue culture has met a great interest in recent years(Leweke and Forkmann, 1982).

To develop a reliable system for anthocyanin synthesis by cell and tissue culture, extensive studies have been reported. Some examples for the results include callus and suspension cultures of *Pyrus communis* spp.(Mehra and Daidka, 1979), *Eucalyptus citriodora*(Ram et al., 1971), sweet potato (Nozue et al, 1987), *Vitis* spp.(Chi and Cormier, 1991 ; Tamura et al., 1989), and carrot(Ozeki and Komamine, 1985 ; Dougall and Volgelien, 1990 ; Volgelien et al., 1990).

It was suggested that modification of micro-environments for the aim of secondary metabolites via cell and suspension cultures are one of the best approaches(Hrazdina, 1982 ; Wijnsma et al., 1986). Among the considerable effects just described above, two major factors such as medium composition and external culture conditions will possibly promise to obtain maximum cell growth as well as high yielding cell lines of anthocyanin(Ernest, 1972).

Nutritional components of various culture media have been demonstrated as essential factors for secondary metabolites production from wide ranges of plant(Dougall and Weyrauch, 1980). Sakuta et al. (1987) have been reported that plant growth regulators and media composition affect not only for cell growth but also for the production of secondary metabolites.

This study was carried out to investigate the effect of various factors on the anthocyanin formation from cell cultures of *Populus alba* X *P. glandulosa*.

MATERIALS AND METHODS

Plant material and cell culture

Primary callus cultures were established from stem cambial tissues of *Populus alba* X *P. glandulosa* microshoots which maintained onto MS(Murashige and Skoog, 1962) medium supplemented with 0.5 mg/l 2, 4-D, 0.1mg/l BAP, 30g/l sucrose, and 7.5 g/l agar for 4 weeks. Callus cultures were placed in a culture room at 25±1℃ under the dark. The medium used for callus proliferation was modified MS medium(MMS) which supplemented with 1.0mg/l 2, 4-D, 0.1mg/l BAP and 30g/l sucrose. Subcultures were followed by every 2 weeks interval. After three months, most friable-looking calli were used for further experiments.

Cell suspension cultures were established by inoculating approximately 1g fresh weight of callus into 100ml Erlenmeyer flasks containing 20ml of MS liquid medium supplemented with 1.0mg/l 2, 4-D, 0.1mg/l BAP, and 30g/l sucrose. These cultures were maintained in 25±1℃ under fluorescent light, agitated at 100rpm/min in gyratory shaker.

Induction of anthocyanin synthesis

To determine optimal conditions for cell growth and anthocyanin producing ability, modified MS media were used. Test of sucrose, phosphate, nitrate, and plant growth regulators were run from 1-10%(w/v), 0-400%, 0-400%(of basic concentration in MS basal medium), and 0-2mg/l 2, 4-D, respectively.

The optimal light intensities were also investigated using Lux meter. Each piece of callus(0.5g fresh weight) was cultured in test tube containing 10ml of the media just mentioned above. To induce high rate of red pigment, the callus cultures were exposed under continuous light conditions for 13 days in a growth chamber which was adjusted to 25±1℃.

After 13 days from initial culture, cell growth was determined as gram fresh weight per test tube and anthocyanin content was measured by U.V. spectrophotometer(Uvikon, USA) at 530 nm with 0.5g fresh weight per test tube.

Induction medium(MS medium with or without 2, 4-D) was tested to stimulate anthocyanin accumulating capacity using 14 day-old suspension cultured cells. Cultures were maintained under the same conditions as specified for the cell suspension culture. This experiment was performed with 3 replications in each of 3 experiments.

Anthocyanin identification

Extraction and identification of anthocyanins were followed by the modified Arditti(1969) methods. Red pigmented callus was harvested from test tube, and dried for 3 days at 50℃ in a dry oven. The pigments were extracted by homogenization and steeping in methanol containing 1% HCl. Because of the high anthocyanin content in certain callus, the extracting fluid was changed every 24 hrs until the tissue became colorless. After drying the extracts, anthocyanins were eluted with absolute methanol. Anthocyanins were precipitated from the methanol with diethylether then redissolved and reprecipitated until the supernatant liquid was clear. The precipitated anthocyanins were dissolved in methanol containing 1% HCl and allowed to stand in a refrigerator for 3-5 days. After move out precipitates by centrifuge, the remained pigments were dissolved in water. This solution was further purified by shaking it with ethyl acetate to remove yellow pigment. After dried, the anthocyanins were redissolved in a minimal amount of methanol containing 1% HCl and stored in a freezer and used for measuring of anthocyanins. The purified anthocyanins were analyzed by thin layer chromatography and U.V. spectrophotometer at 530mm.

RESULTS AND DISCUSSION

Induction of anthocyanin synthesis

In order to investigate suitable culture conditions for high rate of cell growth and anthocyanin accumulation, some factors such as phosphate, nitrate, and sucrose were examined at various levels. These medium components were previously shown to have an influence on the production of secondary metabolites in cell suspension cultures(Knobloch et al., 1982).

The effect of sucrose for cell growth and yield of anthocyanins were investigated. The increment of sucrose concentration at some extend brought a markedly increase in cell growth as well as the amount of anthocyanin(Fig. 1). The highest amount of anthocyanin production per gram fresh weight from cells was obtained using 5% sucrose contained medium. The important role of sugar for the production of anthocyanin was found in sweet potato (Nozue et al., 1987) and *Populus* cell cultures (Matsmoto et al., 1973). It is assumed that these results indicate a close relationship between sucrose and anthocyanin synthesis. The previous results also indicated that the poorly accumulated anthocyanin in the low sugar level may be due to the sucrose depletion.

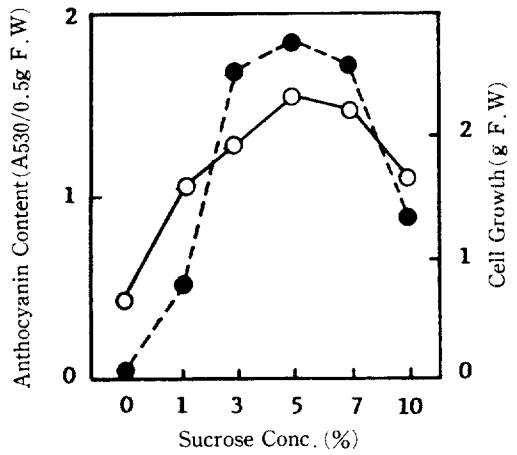

Fig. 1. Effect of sucrose concentrations on growth and anthocyanin production in callus cultures of *Populus alba* X *P. glandulosa*. Calli were cultured on MS agar medium supplemented with 0.5mg/l 2,4-D and 0.1 mg/l BAP under continuous illumination. Cell growth was measured after 13 days of final subcultures.
Cell growth : (○-○),
Anthocyanin content : (●---●)

The amount of NH_4NO_3 concentrations in culture media seems to have effect on anthocyanin formation or growth in *Populus* cells(Fig. 2). High concentration of nitrate was resulted in decrease cell growth and anthocyanin synthesis. At 12.5% nitrate of MS basal medium, anthocyanin production was increased and cell growth also effected at 50% of nitrate concentration in MS medium. Nitrate usually played an important role as stimulating factors for the increment of cell growth and secondary metabolite in higher plants(Dougall and Weyrauch 1980). Yamakawa et al.(1983) reported that anthocyanin production in suspension cultures of *Vitis* is influenced by the inorganic nitrogen on the culture medium. It was shown that high levels of nitrate

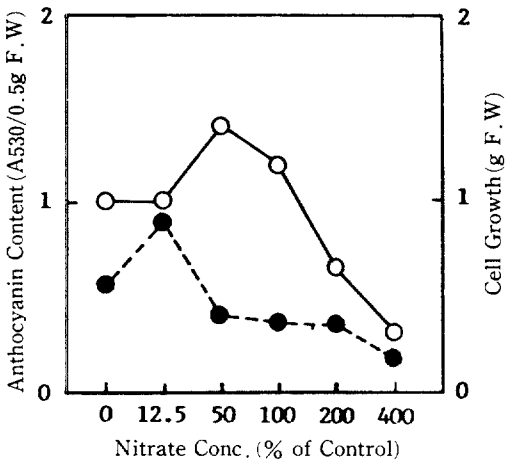

Fig. 2. Effect of nitrate concentrations on growth and anthocyanin production of callus cultures of *Populus alba* X *P. glandulosa*. The ratio of KNO_3 : NH_4NO_3 was 1 : 1.
Cell growth : (○-○),
Anthocyanin content : (●----●)

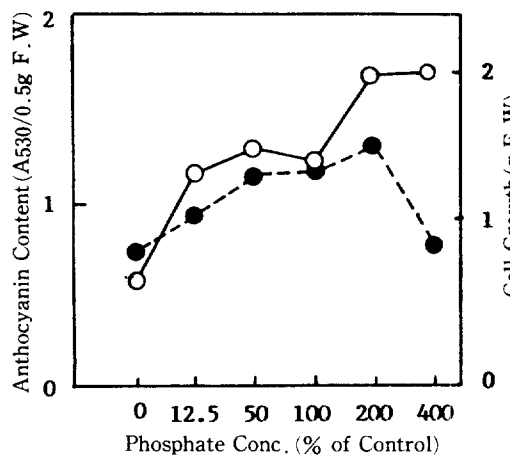

Fig. 3. Effect of phosphate concentrations on growth and anthocyanin production of callus cultures of *Populus alba* X *P. glandulosa*.
Cell growth : (○-○),
Anthocyanin content : (●----●)

dosage in culture medium commonly causes a poor cell growth (Chandler and Dodds, 1983). The correlationship between anthocyanin accumulation and ammonia levels in the medium suggested that ammonia in excess of 3-5 mM in the medium inhibits anthocyanin accumulation (Dougall et al., 1989).

Medium which supplemented with 400% basic phosphate concentration promote cell growth, whereas 200% of basic phosphate concentration in the MS medium effect on anthocyanin formation (Fig. 3). Similar results on low phosphate levels which suppressed anthocyanin synthesis in cell cultures of *Catharanthus roseus* (Knobloch et al., 1982) and *Vitis* (Yamakawa et al., 1983) were reported.

Three components of the production medium such as sugar, nitrate, and phosphate were influenced to the accumulation of anthocyanin in cell cultures.

The effect of several growth regulators (IAA, NAA, 2, 4-D and BAP) on the anthocyanin production were tested. The results obtained are shown in Table 1. With regard to growth regulators, the effect of 2, 4-D was superior to other auxins for rapid growth of callus. Although high concentrations of 2, 4-D markedly inhibited anthocyanin yields, cell growth was superior to other auxins (IAA, NAA) (Table 1). When IAA was incorporated with other

Table 1. Effect of growth regulators on the production of anthocyanin in callus cultures of *Populus alba* X *P. glandulosa*

Growth regulators (Conc.)	2, 4-D		IAA		NAA		BAP	
	AC*	FW**	AC	FW	AC	FW	AC	FW
	***							
0.1	0.93±0.71	0.73±0.23	1.57±0.81	0.73±0.38	0.93±0.55	0.54±0.25	1.05±0.71	0.59±0.45
0.5	0.89±0.30	1.21±0.78	1.79±0.34	0.67±0.55	0.84±0.31	0.78±0.66	0.69±0.55	0.89±0.61
1.0	0.49±0.18	1.51±1.12	2.20±0.81	1.17±0.10	0.75±0.51	1.30±0.91	0.49±0.36	0.67±0.52
1.5	0.33±0.17	1.57±0.92	0.45±0.21	0.35±0.21	0.73±0.71	1.33±0.55	0.52±0.39	0.93±0.88
2.0	0.52±0.20	0.99±0.48	0.81±0.39	0.81±0.39	0.66±0.50	1.01±0.63	0.40±0.45	0.92±0.75

* Anthocyanin content was expressed as absorbance at 530 nm per 0.5g fresh weight.
Abbreviation ; AC : Anthocyanin content

** Increment of fresh weight was expressed from 0.5g fresh weight of initial culture. Cells were investigated 13 days after inoculation. Abbreviation ; FW : Increase of fresh weight

*** Each values represent the mean±SD of 3 replicates each in 3 experiments.

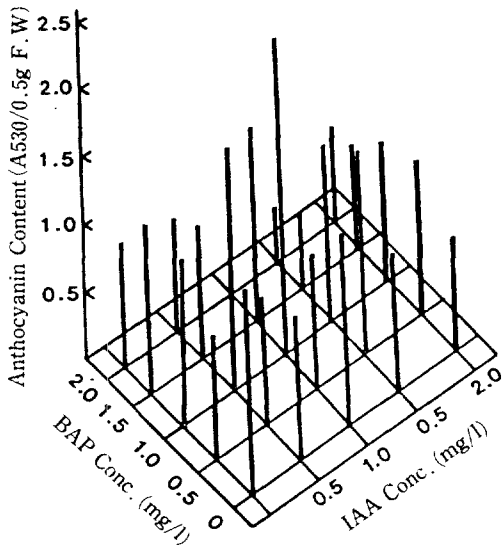

Fig. 4. Effect of IAA vs BAP concentrations on anthocyanin synthesis of *Populus alba* X *P. glandulosa*. Calli were cultured on agar medium supplemented with 3% sucrose under continuous illumination for 14 days.

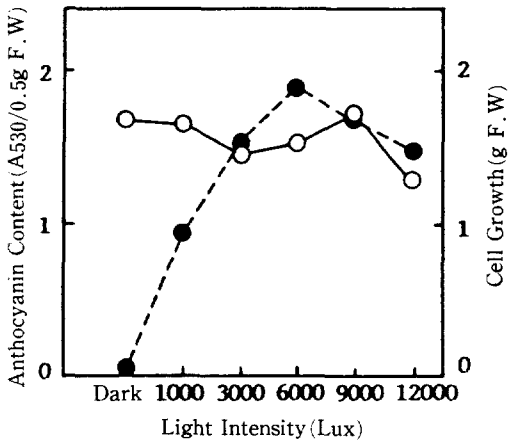

Fig. 5. Effect of light intensities on growth and anthocyanin production of callus cultures of *Populus alba* X *P. glandulosa*.
Cell growth : (○-○),
Anthocyanin content : (●----●)

cytokinins, high anthocyanin producing cells and cell proliferation were found. The examined cytokinin alone did not promote the anthocyanin production.

The best yield of anthocyanin were obtained using MS basal medium with growth regulators combinations of 1.0mg/l IAA and 1.0mg/l BAP (Fig. 4).

Inhibition of anthocyanin accumulation by auxin alone was also reported on cell cultures of *Daucus carrota* (Ozeki and Komamine, 1985), and *Vitis* hybrid (Yamakawa et al., 1983). The studies on the enzymatic control for the induction and suppression suggested that CHS (chalcone synthase) may be the key enzyme in anthocyanin formation which regulated by 2, 4-D in this system, and its activities are reflected in changes of the transcriptional level (Ozeki and Komamine, 1985).

The effect of light on the anthocyanin production are shown in Fig. 5. The sign of anthocyanin synthesis was observed after 24 hrs of irradiation. Maximum level of the light for the pigmentation was 7, 000 lux. Anthocyanin synthesis was not induced in cultured cells which maintained at the dark condition. From this results, we could assume that light exposure is an important factor for the development of anthocyanin in cells. The stimulating effect of light on anthocyanin synthesis has been also investigated (Matsmoto et al., 1973).

When cell suspension cultures of *Populus alba* X *P. glandulosa* which had been transfered to fresh medium and kept in the dark for 48-72 hrs before illuminated with fluorescent light, anthocyanin synthesis started about 48 hrs after illumination, whereas the red pigment was not induced in the cultured cells at the dark (Fig. 6). This strongly suggested that the light exposure is an important stimulator for anthocyanin synthesis.

The cells (10-day-old after subculture in the dark) were transfered to fresh medium supplemented with 0, 0.2, 1.0 and, 2.0mg/l 2, 4-D and then exposed to light. Cell growth was markedly promoted in the medium with 2, 4-D, but, anthocyanin synthesis was inhibited by addition of 2, 4-D in the medium (Table 2). Such observations have led to the proposal that an inverse relationship is exists between culture conditions for growth and secondary metabolite accumulation (Hall and Yeoman, 1986). It is known that high levels of auxins generally inhibit the anthocyanins and other phenolic compounds synthesis in various cell cultures (Constabel et al., 1971).

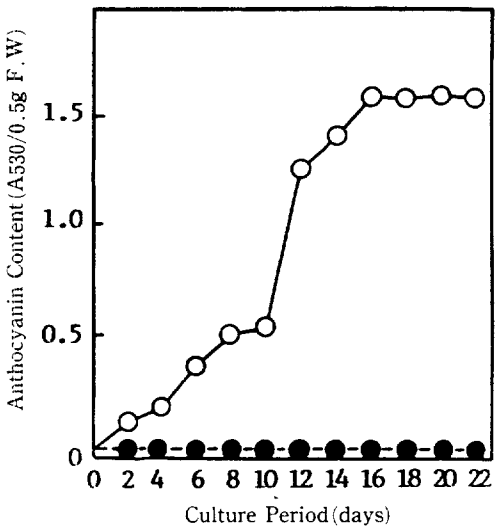

Fig. 6. Light induced anthocyanin synthesis in cell suspension cultures of *Populus alba* × *P. glandulosa*. Cells were cultured on light (○-○) and/or dark (●-●) conditions.

Table 2. Effect of 2,4-D concentrations on anthocyanin synthesis and cell growth in suspension cultures of *Populus alba* X *P. glandulosa*. Cells were cultured on MS liquid medium supplemented with 5% sucrose under continuous illumination for 14 days.

Conc. of 2,4-D	Cell growth (g F.W)*	Anthocyanin content (A530/g F.W)
0	0.85±0.21**	1.41±0.5
0.5	4.52±1.01	0.95±0.17
1.0	5.62±1.05	0.79±0.21
2.0	5.01±0.96	0.61±0.39

* Increment of fresh weight was measured after filterated by Watman No. 2.

** Each values represents the mean±SD of 3 replicates.

Anthocyanin identification

Anthocyanin was identified by TLC with four solvent systems and U.V. spectrophotometer (Table 3). Using different solvent system, the presence of one single major trace from two others could be ascertained. Spectral measurements of MeOH-HCl extracts of these lines showed max values at 507nm indicating that the anthocyanins derived from callus might be pelargonidin derivates. In agreement with spectral data, chromatography analysis yielded the pelargonidin aglycone.

Table 3. Rf values and spectral data of purified anthocyanin from the callus of *Populus alba* X *P. glandulosa*

Pigment	Rf values in solvent* (Rf×100) A	B	C	D	Color	Max**
Purified anthocyanin	45	26	37	70	red	507
Pelargonidin 3-rhamnoside 5-glucoside	46	24	39	70	red	505

* Mark Kieselgel 60F254 were used descending 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)

** Spectral data were determined in methanol containing 0.01% HCl

In this results, such a feature of cell metabolism must be taken into consideration not only when selecting suitable physical and chemical conditions for culture but also when designing the actual culture system itself.

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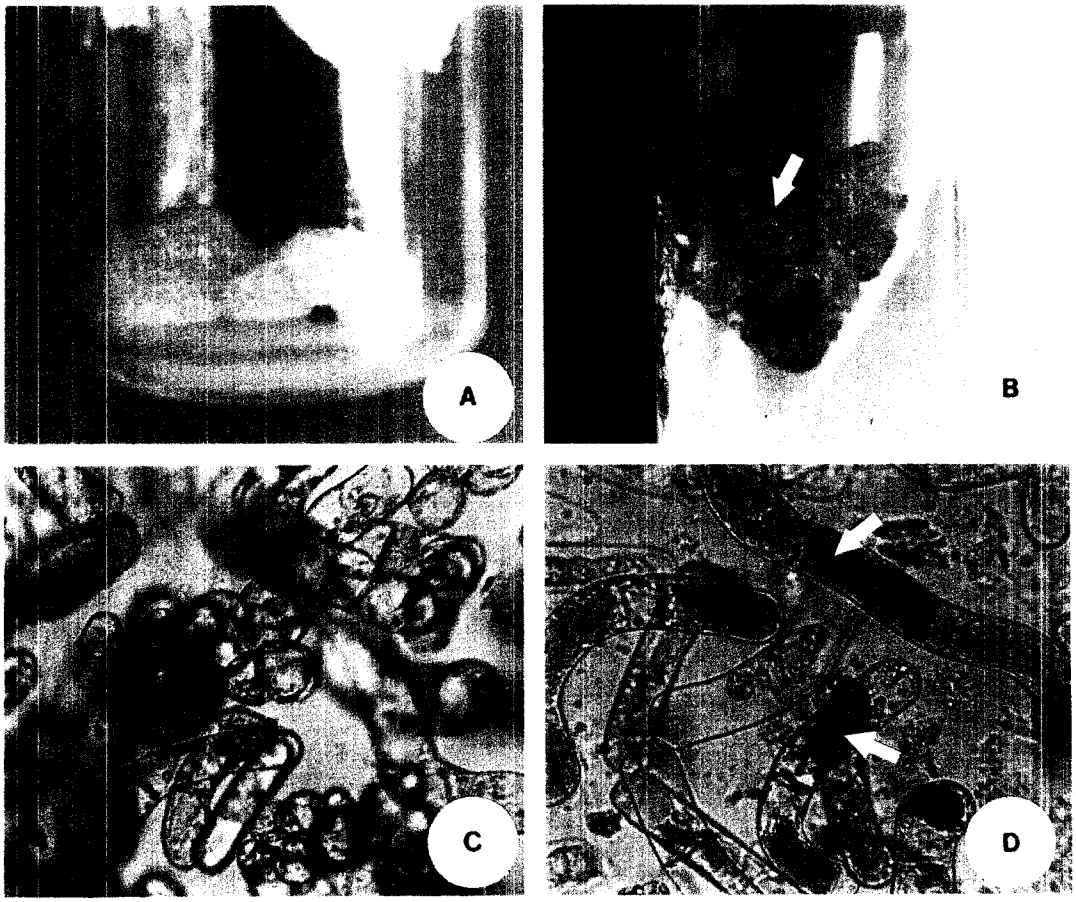

Fig. 7. Anthocyanin synthesis in callus and suspension cultured cells of *Populus alba* X *P. glandulosa*.
A : Callus cultures on MS basal medium supplemented with 1.0mg/l 2,4-D and 0.1mg/l BAP.
B : Pigmentation of callus on product medium under the continuous light condition of 7,000 lux (arrow indicates high pigmentation).
C : Suspension cultured cells in liquid MS basal medium containing 0.5mg/l 2,4-D and 0.1mg/l BAP.
D : Pigmentation of suspended cells on MS liquid medium containing 1.0mg/l IAA and 1.0mg/l BAP under the continuous light condition of 7,000 lux (arrows indicate cells with pigmentation).

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