J. Plant Biotechnology(2002) *Vol. 4(2). pp. 77~82*

Journal of Plant Biotechnology

Effects of 2,4-D, BA, and Sucrose on Growth, Production of Anthocyanin, pH, and Sugar Content in 'Sheridan' Grape Cell Suspension Cultures

Seung-Heui Kim, Seon-Kyu Kim*

Dept. of Horticulture, Chungbuk National University, Cheongju, 361-763, Korea

Key words: Callus culture, cell growth, fresh weight, *in vitro*.

Abstract

To elucidate the effect of sucrose on cell growth and anthocyanin production, 1, 3, 5, and 7% sucrose were applied to liquid MS basal medium supplemented with 0.5 mg/L BA + 0.1 and 1 mg/L 2,4-D. Higher sucrose concentration decreased the cell growth regardless of the hormonal composition. Gain in fresh weight was gradual, showing the peak at day 12 in culture, and then decreased. Anthocyanin content increased with sucrose concentration in the medium, and practically there was no difference in anthocyanin content between the two media differing in 2,4-D content. Sucrose concentration for appropriate anthocyanin production was 7%, while 5% was more suitable for increase in total anthocyanin content. At higher sucrose levels, anthocyanin content was high due to the cessation of the cell growth. Medium pH decreased at the early stage and gradually increased thereafter.

Introduction

Plant cell culture is a useful method to get secondary plant metabolites such as alkaloids, terpenoids, and flavonoids. There have been many reports on anthocyanin production in cultured plant cells (Yamamoto et al., 1989).

Many secondary metabolites accumulate in specific tissues and cells of higher plants, or at specific stages during the growth of cultured cell. The accumulation of most secondary metabolites in cultured cell is maximal during the stationary phase of growth, for example, the accumulation of anthocyanin in suspension cultures of *Daucus carota* (Noe et al., 1980) and of *Catharanthus roseus* (Hall and Yeoman, 1986), and the accumulation of DOPA in callus cultures of *Stizolobium hassjoo* (Obata-Sasamoto and Komamine, 1983). However, Berlin et al. (1986) reported peaks of accumulation of betalains during the logarithmic phase in suspension cultures of *Chenopodium rubrum*.

Many attempts have been made to produce useful secondary metabolites using plant cell cultures, but most have been unsuccessful, because the activity of cells for synthesis of secondary products is lost in most cases when the cells are dedifferentiated and grow rapidly in cultures (Ozeki and Komamine, 1981). Recently, cell lines which synthesize secondary products at a high yield have been reported to be obtained by selection. In cultures of *Daucus carota*, it has been reported that some cell lines can synthesize anthocyanin (Sugano and Hayashi, 1967; Ibrahim et al., 1971; Alfermann and Reinhard, 1971; Schmitz and Seitz, 1972; Dougall and Weyrauch, 1980), but such cell lines were also obtained by spontaneous selection. Kinnersley and Dougall (1980) reported success in increasing the anthocyanin yield. They used a method based on cell-aggregate size with a wild carrot cell culture.

Color is directly related to quantity and quality of the anthocyanin in the skin of the fruit (Webb, 1970). These pigments, in order of their stability, consist of mono- or diglucoside forms of malvidin, peonidin, petunidin, cyanidin, and delphinidin (Flora, 1978; Hrazdina et al., 1970;

* Corresponding author, E-mail; kimskyu@cbu.ac.kr
Received Apr. 14, 2002; accepted May. 16, 2002.

Simpson et al., 1976). Several reports indicate that malvidin mono- and diglucosides are the most stable for red color (Ballinger et al., 1974), and that acylation of anthocyanin molecules increases their stability. As a species, *V. vinifera* is unique in having only anthocyanin monoglucosides and their acylated forms (Ribéreau-Gayon, 1968). By contrast, *V. rotundifolia* (muscadine grape) has only nonacylated anthocyanin diglucosides (Ribéreau-Gayon, 1982) and apparently does not produce acylated anthocyanin. Most modern red *V. vinifera* wine grapes are high in malvidin and acylation products. In comparison, *V. rotundifolia* forms are relatively low in malvidin (Goldy, 1986).

Enhancement of anthocyanin productivity can be affected by different factors such as light (Siegelman and Hendricks, 1958; Jackson, 1980; Downs et al., 1965; Arakawa et al., 1985; Kubo et al., 1988), nitrogen source (Sargent and King, 1974; Gamborg et al., 1968; Tal and Goldberg, 1982; Yamamoto et al., 1989), type of sugar (Matsumoto et al., 1970; Sato et al., 1996; Do and Cormier, 1991; Hong et al., 1989; Schlatmann et al., 1994), temperature (Zhang et al., 1997; Morris, 1986; Sahai and Shuter, 1984), and phytohormone conditions (Mori et al., 1993; Mori and Sakurai, 1994; Ozeki and Komanine, 1986; Kinnersley and Dougall, 1980; Lee and Wetzstein, 1988).

The present study was conducted to elucidate the effects of 2,4-D, BA, and sucrose level on cell growth and anthocyanin production with the ultimate goal for the mass production of anthocyanins through cell suspension cultures in 'Sheridan' grapes.

Materials and Methods

Plant materials

One-eye cutting of 12 cm-long 'Sheridan' grapes were cut on sand bed and placed in a greenhouse for 4 weeks. Shoot tips and nodal segments were dissected from shoots, and the explants were surface sterilized in 1% sodium hypochlorite solution containing 3 drops of Tween-20 for 15 min, and then rinsed 3 times with sterile distilled water.

Terminal and axillary bud explants of ca. 0.6 cm were excised and then individually transferred into test tube containing 20 mL of culture medium comprised of 1/2 Murashige and Skoog (MS) with 3% sucrose and 0.2% gelrite.

The culture conditions were maintained at 25 ± 1°C with a 16-hour photoperiod under 40 μmol · m^{-2} · s^{-1} photosynthetic photon flux (PPF) provided by fluorescent lamps.

Callus induction

Young, fully expanded leaves, petiole, and stem sections of 'Sheridan' grape were collected from in vitro-grown cultures. The leaves were aseptically sliced into 10 x 10-mm square pieces, discarding their margin. Longitudinally-sectioned stems were placed to medium with cut surface down. Five explants were placed in each 90 x 15-mm petri dish containing 20 mL of MS medium supplemented with 1 mg/L NAA + 1 mg/L BA. In all media, 8 g/L Bacto-agar(DIFCO) and 3% sucrose were used and the pH of all media was adjusted to 5.8 with 0.2N KOH before autoclaving.

Callus and cell suspension culture

Induced calli were transferred periodically to freshly prepared culture medium: MS supplemented with 1.0 mg/L 2,4-D, 0.5 mg/L BA, 3% sucrose and 0.2% gelrite. The stock cultures were subcultured every 4 weeks under complete darkness.

The cell cultures were passed through nylon screens of 420 μm pore size. This sieving procedure was performed at every subculture, and cultures subcultured more than 5 times were used in the experiments. Induction of the cell suspension culture was obtained by inoculating callus cells into 250 mL shaking flasks containing 70 mL of the liquid medium (with 2% sucrose without agar) and followed by incubation on a gyratory shaker (100 rpm, 5 cm) at 25 ± 1°C under complete darkness. The suspension cultures were also subcultured periodically into the liquid medium with an inoculum size of 10 to 15%.

Sucrose concentration

To examine the influence of sucrose level on cell growth and anthocyanin content, 20 g/L fresh cell which had been subcultured several times with 20 g/L sucrose, 1.0 mg/L 2,4-D + 0.5 mg/L BA in 250 mL flask under complete darkness were cultured on basal MS medium. The concentrations of sucrose were adjusted to 1, 3, 5, and 7%.

Growth

Samples were collected every 4 days for 16 days. Cells were separated from the culture medium by filtration through filter paper and weighed. Results were expressed as fresh cell weight (FCW. g) per flask.

Residual sugar in medium

Samples were collected every 4 days for 16 days. After filtering, the residual medium was used for sugar analysis. The levels of fructose and glucose in liquid medium were analyzed by high performance liquid chromatography (HPLC 2690, Alliance System, USA) with high performance carbohydrate column and refractive index detector (Waters, USA). A mobile phase consisting of 80% acetonitrile was used at a flow rate of 1.0 mL · min^{-1}.

Anthocyanin content

Samples were collected every 4 days for 16 days. Fresh cells were extracted overnight using a solution containing 1 g/L HCl-methanol at 4℃. After centrifugation at 100 × g for 5 min, the absorbance of the clear supernatant was measured. Optical density of the extract solutions was measured at 530 nm with a spectrophotometer (UVIKON 930, Uvikon, USA). Anthocyanin content was calculated with the extinction coefficient ($E^{1\%}_{1cm}$= 566 at 530 nm) of cyanidin-3-glucoside (Extrasynthese) in the same solvent. Results were expressed as μg/g fresh weight of cells.

Medium pH

Samples were collected every 4 days for 16 days. After filtering, the pH of medium was measured by pH meter (Triode™, Orion, Co.) at 25℃.

Results and Discussion

To investigate the effect of sucrose on cell growth and anthocyanin production, 1, 3, 5, and 7% sucrose were applied to MS basal medium supplemented with 0.5 mg/L BA + 0.1 or 1 mg/L 2,4-D. Higher sucrose concentration decreased the cell growth regardless of the hormonal composition and combination. Fresh weight gain was gradual, showing the peak at day 12 in culture, and then decreased. In 0.5 mg/L BA + 1.0 mg/L 2,4-D combination, 3% sucrose resulted in sharp increase (Figure 1). The decrease of cell growth in media containing a high concentration of sucrose may have been caused by inhibition of nutrient uptake due to an increase in the osmotic potential or the high viscosity of the medium (Sato et al., 1996).

Anthocyanin content increased with sucrose concentration in the medium, and practically there was no difference in anthocyanin content between the two media. Anthocyanin content was the highest with 0.5 mg/L BA + 1.0 mg/L 2,4-D and 7% sucrose after 12 days in culture, and then decreased. Anthocyanin content was low with 3% sucrose which favored the growth in both media (Figure 2).

Sucrose concentration for appropriate anthocyanin production was 7%, while 5% was more suitable for increase in total anthocyanin content. Significant differences among sucrose level were not noted with 0.5 mg/L BA + 1.0 mg/L 2,4-D. The combination also showed the peak after 12 days in culture and then decreased.

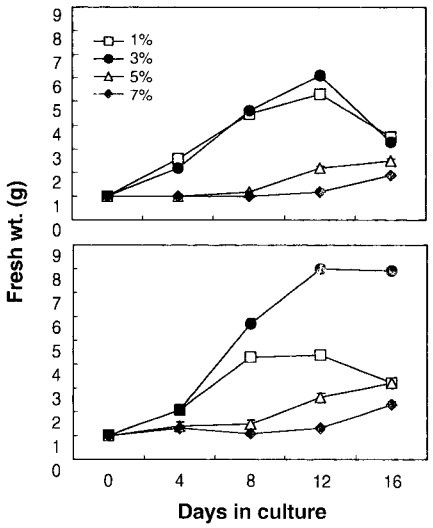

Figure 1. Fresh weight of 'Sheridan' grape as affected by sucrose level in liquid MS medium supplemented with 0.5 mg/L BA + 0.1 mg/L (upper) and 1.0 mg/L 2,4-D (lower) under light. Bars represent ± SE.

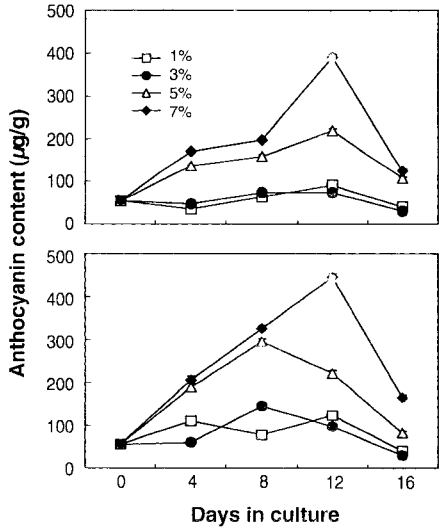

Figure 2. Anthocyanin content of 'Sheridan' grape as affected by sucrose level in liquid MS medium supplemented with 0.5 mg/L BA + 0.1 mg/L (upper) and 1.0 mg/L 2,4-D (lower) under light. Bars represent ± SE.

Anthocyanin content increased significantly in 0.5 mg/L BA + 1.0 mg/L 2,4-D combination with 3% sucrose, especially during 4 to 8 days in culture. This was thought to be the result of vigorous cell growth (Figure 3). At higher sucrose levels, anthocyanin content was high due to the cessation of the cell growth. Slowing and killing of cells occurred from the beginning of the culture. Anthocyanin content at low sucrose levels was somewhat low due to the vigorous cell growth.

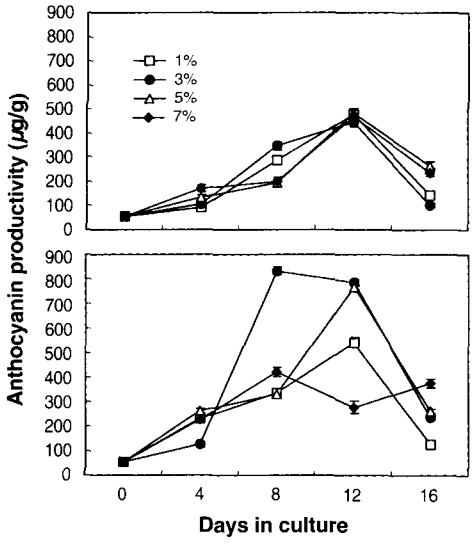

Figure 3. Anthocyanin productivity of 'Sheridan' grape as affected by sucrose level in liquid MS medium supplemented with 0.5 mg/L BA + 0.1 mg/L (upper) and 1.0 mg/L 2,4-D (lower) under light. Bars represent ± SE.

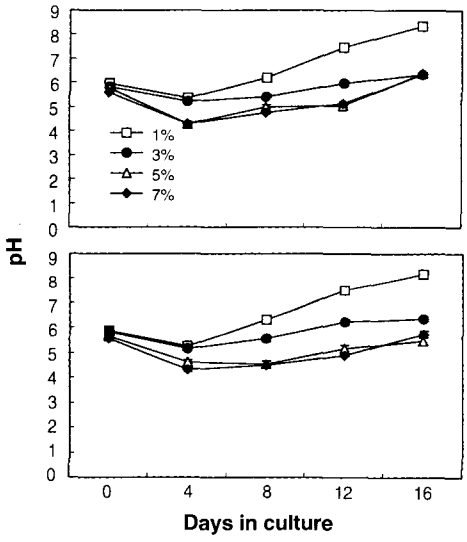

Figure 4. Changes in pH of MS medium supplemented with 0.5 mg/L BA + 0.1 mg/L (upper) and 1.0 mg/L 2,4-D (lower) with varying sucrose levels in suspension cultures of 'Sheridan' grape under light. Bars represent ± SE.

The medium pH decreased at the early stage (after 4 days in culture) and gradually increased thereafter in both media (Figure 4). The best performance of cell growth and anthocyanin production was observed at pH 7~8 after 12 days in culture.

The presence of a high concentration of sucrose in the culture medium could induce a considerable increase in the carbohydrate content of the cells, as was shown in *Catharanthus roseus* cells (Schlatmann et al., 1994). A sucrose concentration of 9.9% and 8% greatly enhanced anthocyanin production in *Vitis* cell culture (Yamakawa et al., 1983), and a 5% sucrose concentration enhanced anthocyanin accumulation in *Populus* (Matsumoto et al., 1970) and *Euphorbia milli* (Yamamoto et al., 1989) suspension cultures. This experiment showed that high anthocyanin productivity and anthocyanin content were obtained at respective 2% and 7% sucrose.

Acknowledgment

This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Research Center for the Development of Advanced Horticultural Technology (HortTech) at Chungbuk National University.

References

Alfermann W, Reinhard E (1971) Isolierung anthocyanhaltiger und anthocyanfreier Gewebestamme von *Daucus carota*: Einfluss von Auxinen auf die Anthocyanbildung. Expe rimentia 27: 353-354.

Arakawa O, Hori Y, Ogata R (1985) Relative effectiveness and interaction of ultraviolet-B, red and blue light in anthocyanin synthesis of apple fruit. Physiol Plant 64: 323-327.

Ballinger WE, Maness EP, Makus AJ, Carroll DE Jr (1974) A comparison of anthocyanins and wine color quality in black grapes of 39 clones of *Vitis rotundifolia* Michx. J Amer Soc Hort Sci 99: 338-341.

Berlin J, Sieg S, Strack D, Bokern C, Harms C (1986) Production of betalains by suspension cultures of *Chenopodium rubrum* L. Plant Cell Tissue Organ Culture 5: 163-174.

Do CB, Cormier F (1991) Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape (*Vitis vinifera* L.) cell suspension. Plant Cell Rep 9: 500-504.

Dougall DK, Weyrauch KW (1980) Growth and anthocyanin production by carrot suspension cultures grown under

chemostat conditions with phosphate as the limiting nutrient. Biotechnol Bioeng 22: 337-357.

Downs RJ, Siegelman HW, Butler WL, Hendricks SB (1965) Photoreceptive pigments for anthocyanin synthesis in apple skin. Nature 205: 909-910.

Flora LF (1978) Influence of heat, cultivar and maturity on the anthocyanidin-3,5-diglucosides of muscadine grapes. J Food Sci 43: 1819-1821.

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158.

Goldy RG, Ballinger WE, Magness EP (1986) Fruit anthocyanin content of some *Euvitis* × *Vitis rotundifolia* hybrids. J Amer Soc Hort Sci 111: 955-960.

Hall RD, Yeoman MM (1986) Temporal and spatial heterogeneity in the accumulation of anthocyanins in cell cultures of *Catharanthus roseus* (L.) G Don. J Exp Bot 145: 1055-1065.

Hong YC, Read PE, Harlander SK, Sabuza TP (1989) Development of a tissue culture system for immature strawberry fruit. J Food Sci 54: 388-392.

Hrazdina G, Borzell AJ, Robinson WB (1970) Studies on the stability of the anthocyanidin-3,5-diglucosides. Amer J Enol Vitic 21: 201-204.

Ibrahim RK, Thakur ML, Permanand B (1971) Formation of anthocyanins in callus tissue cultures. Lloydia 34: 175-182.

Jackson JE (1980) Light interception and utilization by orchard systems. In: J Janick(Ed.). Horticultural Reviews. Vol 2. AVI Publ. Co., Westport, Conn.

Kinnersley AM, Dougall DK (1980) Increase in anthocyanin yield from wild-carrot cell cultures by a selection system based on cell aggregate size. Planta 148: 200-204.

Kubo Y, Taira S, Ishio S, Sugiura A, Tomana T (1988) Color development of four apple cultivars grown in the Southwest of Japan, with special reference to fruit bagging. J Jap Soc Hort Sci 57: 191-199.

Lee N, Wetzstein HY (1988) Protoplast isolation and callus production from leaves of tissue-cultured *Vitis* spp. Plant Cell Rep 7: 531-534.

Matsumoto T, Nishida K, Noguchi M, Takami E (1970) Isolation and identification of an anthocyanin from the cell suspension culture of poplar. Agric Biol Chem 34: 1110-1114.

Mori T, Sakurai M, Shigeta J, Yoshida K, Kondo K (1993) Formation of anthocyanins from cells cultured from different parts of strawberry plants. J Food Sci 58: 788-793.

Mori T, Sakurai M (1994) Production of anthocyanin from strawberry cell suspension cultures: effects of sugar and nitrogen. J Food Sci 59: 588-593.

Morris P (1986) Regulation of product synthesis in cell cultures of strawberry. Effect of culture temperature. Plant Cell Rep 5: 427-429.

Noé W, Langebartels C, Scitz HU (1980) Anthocyanin accumulation and PAL activity in a suspension culture of *Daucus carota* L. Inhibition by L-α-aminooxy-β-phenylpropionic acid and γ-cinnamic acid. Planta 149: 283-287.

Obata-Sasamoto H, Komamine A (1983) Effect of culture conditions on DOPA accumulation in a callus culture of *Stizolobium hassjoo*. Planta Med 49: 120-123.

Ozeki Y, Komamine A (1981) Induction of anthocyanin synthesis in relation to embryogenesis in a carrot suspension culture: Correlation of metabolic differentiation with morphological differentiation. Physiol Plant 53: 570-577.

Ozeki Y, Komanine A (1986) Effects of growth regulators on the induction of anthocyanin synthesis in carrot suspension cultures. Plant Cell Physiol 27: 1361-1368.

Ribéreau-Gayon P (1968) Les composés phénoliques des végétaux. Ch. VI. Les anthocyanes. Dunod Paris pp 142-172.

Ribéreau-Gayon P (1982) The anthocyanins of grapes and wines. In: P Markakis(Ed.). Anthocyanins as food colors. Academic Press. New York pp 209-230.

Sahai OP, Shuter ML (1984) Environmental parameters influencing phenolics production by batch cultures of *Nicotiana tabacum*. Biotech Bioeng 26: 111-120.

Sargent PA, King J (1974) Investigations of growth promoting factors in conditioned soybean root cells and on the liquid medium in which they grow: ammonium, glutamine and amino acids. Can J Bot 52: 1747-1755.

Sato K, Nakayama K, Shigeta JI (1996) Culturing conditions affecting the production of anthocyanin in suspended cell cultures of strawberry. Plant Sci 113: 91-98.

Schlatmann JE, Fonck E, Ten Hoopen HJG, Heijnen JJ (1994) Plant Cell Rep 14: 157-160.

Schmitz M, Seitz U (1972) Hemmung der Anthocyansynthese durch Gibberellinsäure A_3 bei Kalluskulturen von *Daucus carota*. Z Pflanzenphysiol 68: 259-265.

Siegelman HW, Hendricks SB (1958) Photocontrol of anthocyanin synthesis in apple skin. Plant Physiol 33: 185-190.

Simpson KL, Lee TC, Rodriguez DB, Chichester CO (1976) Metabolism in senescent and stored tissues pp. 779-842. In: TW Goodwin(Ed.). Chemistry and biochemistry of plant pigments. Academic Press, New York

Sugano N, Hayashi K (1967) Dynamic interrelation of cellular ingredients relevant to the biosynthesis of anthocyanin during tissue culture of carrot aggregen. Studies on anthocyanins LVII. Bot Mag Tokyo 80: 440-449.

Tal B, Goldberg I (1982) Growth and diosgenin production by *Dioscorea deltoidea* cells in batch and continuous cultures. Planta Med 44: 107-110.

Webb AA (1970) Anthocyanin pigments in grapes and wines. Suomena Kemistilehti 43: 67-74.

Yamakawa T, Ishida K, Kato S, Kodama T, Minoda Y (1983) Formation and identification of anthocyanins in cultured cells of *Vitis* spp. Agric Biol Chem 47: 997-1001.

Yamamoto Y, Kinoshita Y, Watanabe S, Yamada Y (1989) Anthocyanin production in suspension cultures of high producing cells of *Euphorbia millii*. Agric Biol Chem 53: 417-423.

Zhang W, Seki M, Furusaki S (1997) Effect of temperature and its shift on growth and anthocyanin production in suspension cultures of strawberry cells. Plant Sci 127: 207-214.