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Effects of Sucrose Level and Nitrogen Source on Fresh Weight and Anthocyanin Production in Cell Suspension Culture of 'Sheridan' Grape (*Vitis* spp.)

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Abstract

To establish an *in vitro* mass production system of grape anthocyanin pigments through callus and cell suspension culture, the effects of nitrogen source and sucrose on fresh weight and anthocyanin production in cell suspension culture of 'Sheridan' grape level were studied. When the medium was devoid of NO_3^-, cell fresh weight was either remained stable (1% sucrose) or slightly decreased with culture time (2, 3, and 4% sucrose). When NH_4^+ was lacking, 3% sucrose was most favorable for cell growth. When NH_4^+ was supplied as N source, the anthocyanin content of 2% sucrose containing medium was maintained 2 times higher than other levels till day 8 in culture, then that of 3 and 4% sucrose which peaked at day 12 thereafter. The anthocyanin content was low than NO_3^--free media. Total anthocyanin content in NH_4^+-free medium was just about a half of that of NH_4^+ medium. Anthocyanin production of 2% sucrose in NH_4^+ medium was maintained about 3-fold till day 8, then decreased thereafter. In NH_4^+ medium, pH decreased gradually with final pH of 3.5 to 4.0, while pH in NH_4^+-free medium increased with final pH of 6.5 to 7.5.

Introduction

Anthocyanins, a group of flavonoids widely distributed among plant species, have been well documented as a tinctorial additives to foods (Gao and Mazza, 1996).

Anthocyanin is fundamentally responsible for all the color differences between grapes and the resultant wines (Ribreau-Gayon, 1982). It was well-known that the distribution of anthocyanins in the fruit is complex and varies according to species, variety, maturity, seasonal conditions, production area, and yield of fruit (Mazza and Miniati, 1993).

Plant cell cultures provide an attractive route to produce high-value plant-derived products, such as flavors, fragrances, alkaloids, colorants and pharmaceuticals that are very expensive to synthesize chemically and that occur naturally only at very low concentrations (Zhang et al., 1998). With high demand of food colorants from natural sources instead of synthetic materials, anthocyanin production has been reported in different plant tissue or cell cultures (Zhang et al., 1997).

There have been many reports on anthocyanin production in cultured plant cells: sweet potato (Nozue et al., 1987), grape (Yamakawa et al., 1983; Tamura et al., 1989), and strawberry (Hong et al., 1989). The activity of cells for synthesis of secondary products is lost in most cases when the cells are differentiated and grow rapidly in cultures (Ozeki and Komamine, 1981). Anthocyanin production is influenced by different factors such as UV, light, nitrogen source, type of sugar, osmotic stress, temperature, elicitor conditioning and phytohormone conditions (Zhang et al., 1998). In *Vitis vinifera* cell suspension, it was noted that induction of high anthocyanin production was obtained following an osmolality raise of the growth medium provoked by an high sugar concentration, and as a conse-

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quence of a low nitrate concentration of the culture medium (Cormier et al., 1990). Such anthocyanin-promoting conditions were shown to be detrimental to biomass proliferation and the main anthocyanin accumulated was peonidin 3-glucoside.

Recent restriction of the use of synthetic red dyes in food has activated more research on plant pigments. Production of natural anthocyanin pigments by plant cell cultures as red food-coloring agents has been suggested as alternative to synthetic dyes (Zhang et al., 1998). And it has been proposed that regular consumption of red wine in moderate amounts reduces the risk for coronary heart disease via protection of LDL against oxidative damage and via inhibition of platelet aggregation (Fuhrman et al., 1995; Nigdikar et al., 1998; Renaud and de Lorgeril, 1992).

Among factors affecting anthocyanin production in grape suspension cultures, many works were done with N source in combination with sucrose due to easy manipulation. A medium with reduced nitrate and elevated sucrose induced high accumulation of anthocyanins (Do and Cormier, 1991; Decendit and Merillon, 1996). Until now, most of the works were performed with European grape (*Vitis vinifera*) though almost all of our vineyards in korea are actually planted with hybrid grapes.

The objective of this research was to evaluate the optimal conditions for obtaining highest anthocyanin production and/or cell growth by combining two variables, nitrogen source and sucrose level, in callus and cell suspension culture of hybrid grape 'Sheridan'.

Materials and Methods

Plant materials

One-eye cutting of 12 cm-long 'Sheridan' grapes were rooted 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℃ 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 1.0-cm^2 square pieces, discarding their margin. Longitudinal-sectioned stems were placed to medium with cut surface down. Five explants were placed in each 9.0-cm petridish containing 20 mL of MS medium supplemented with 1 mg/L NAA + 1 mg/L BA. In all media, 8 g/L Bactoagar (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℃ under complete darkness. The suspension cultures were also subcultured periodically into the liquid medium with an inoculum size of 10 to 15%.

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.

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 ($E^{1\%}_{1\ cm}$= 566 at 530 nm) of

cyanidin-3-glucoside (Extrasynthese) in the same solvent. Results are expressed as $\mu g/g$ fresh weight of cells.

Medium pH

Samples were collected every 4 days till 16 days. After filtering, the pH of medium was measured by pH meter (Triode™, Orion, Co.) at 25±1℃.

Results and Discussion

When NH_4^+ was the sole N source, cell fresh weight was remained stable (1% sucrose) or slightly decreased with culture time (2, 3, and 4% sucrose) (Figure 1). Above 2% sucrose, the cell growth decreased and cells were mostly killed. Most cell culture except for 1% sucrose eventually died. On the other hand, when NO_3^- was the sole N source, 3% sucrose was most favorable for cell growth, surpassing other sucrose concentrations. With sucrose concentrations which favored cell growth (1 and 3%), a sharp increase in cell fresh weight was observed from day 4, peaked at day 12 in culture, then decreased thereafter. NO_3^- was more effective for cell growth, than NH_4^+ and the difference was highly significant. Three percent sucrose markedly increased the cell the growth (Figure 1).

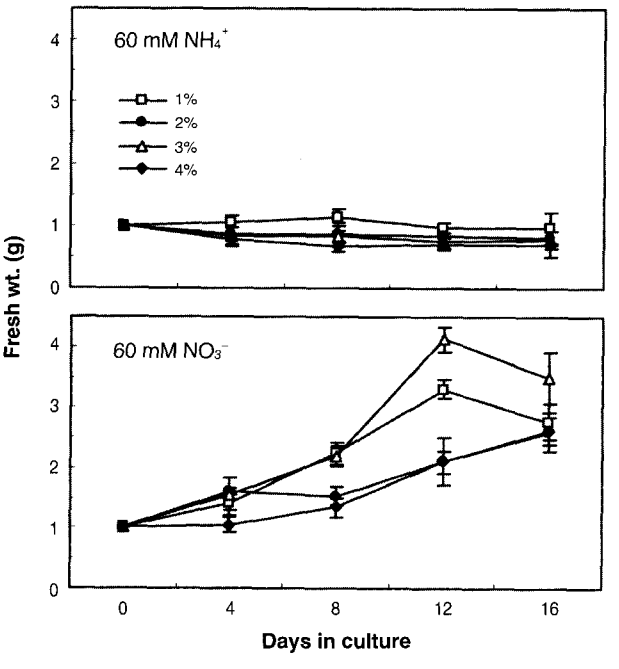

Figure 1. Fresh weight of 'Sheridan' grape cell suspension cultures in MS medium containing 60 mM NH_4^+ and NO_3^- each with varying sucrose level. Bars represent ± SE.

When NO_3^- was reduced from 25 mM to 6.25 mM in NH_4^+ containing media, the growth was decreased (Do and Cormier, 1991).

When NH_4^+ was supplied as N source, anthocyanin content of 2% sucrose containing medium was maintained 2 times higher than other levels till day 8 in culture, then that of 3 and 4% sucrose which peaked at day 12 surpassed thereafter. Anthocyanin content was lower than NO_3^--free media, and there were no significant differences among other treatments (Figure 2).

As was in NH_4^+-medium, 2% sucrose was most effective for anthocyanin production in NO_3^- containing medium. But total anthocyanin content in NO_3^- containing medium was about 50% lower than of that of NH_4^+ medium. Anthocyanin production of 2% sucrose in NH_4^+ medium was maintained about 3-fold till day 8 then decreased thereafter (Figure 2).

In contrast to anthocyanin content, total anthocyanin production linked with growth in NO_3^- medium was much higher than that of NH_4^+ medium. Anthocyanin production increased linearly with culture duration in all sucrose levels except for 1% that peaked at day 12 (Figure 3). NO_3^-

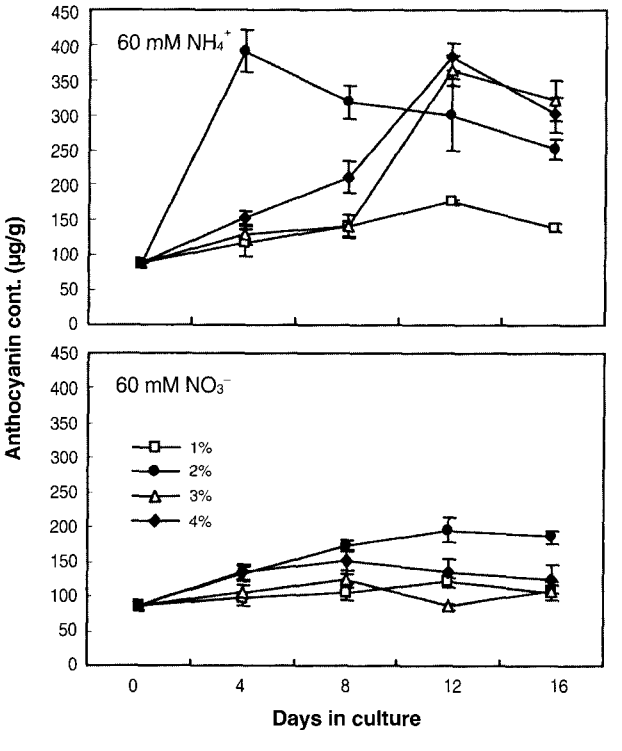

Figure 2. Anthocyanin content of 'Sheridan' grape cell suspension cultures inoculated in medium containing 60 mM NH_4^+ and NO_3^- each with varying sucrose level. Bars represent ± SE.

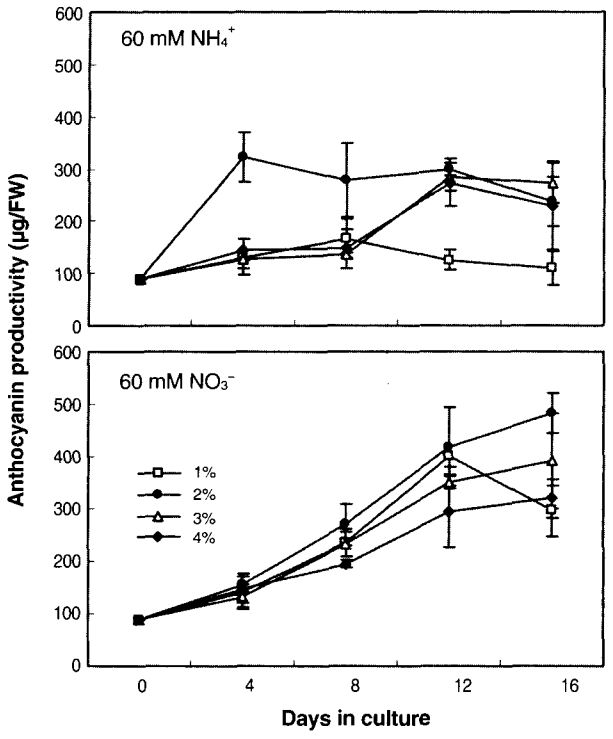

Figure 3. Anthocyanin productivity of 'Sheridan' grape cell suspension cultures in MS medium containing 60 mM NH_4^+ and NO_3^- each with varying sucrose level. Bars represent ± SE.

media that favored the cell growth resulted in anthocyanin content decrease while NO_3^--free media increased the anthocyanin content but the cells were thought to be dead.

In NH_4^+ medium, pH decreased gradually with final pH of 3.5 to 4.0, while pH in NO_3^- medium increased gradually with final pH of 6.5 to 7.5 (not shown). The best performance of cell growth was observed in pH 4~5 after 8 days in culture, while anthocyanin production reached a peak in pH 3~4 at day 12 except for 2% sucrose concentration (Figure 1 and 2).

Anthocyanin production in suspension cultures of hybrid *Vitis* was shown to be influenced by the low inorganic nitrogen and high sucrose concentration of the culture medium (Yamakawa et al., 1983). Tal et al. (1982) demonstrated that the carbon and nitrogen concentrations were important factors influencing diosgenin production in *Dioscorea deltoidea*. Increasing the sucrose concentration decreased the cell density and increased the anthocyanin content (Sato et al., 1996). Yamakawa et al. (1983) reported that high sucrose and low phosphate concentrations brought about a marked increase of anthocyanin formation, while high concentrations of nitrate, phosphate, and 2,4-D repressed the pigment formation. Our results show that nitrate and sucrose influence the anthocyanin production, and support the argument for antagonistic regulation of growth and secondary metabolism of plant cell cultures.

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