J. Plant Biotechnology(2002) *Vol. 4(2). pp. 83~89*

Journal of Plant Biotechnology

Effect of Nitrogen Source on Cell Growth and Anthocyanin Production in Callus and Cell Suspension Culture of 'Sheridan' Grapes

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Key words: Fresh weight, medium pH, nitrogen amount, NH_4^+, NO_3^-, sugar content.

Abstract

To establish *in vitro* mass production system of grape anthocyanin pigments through callus and cell suspension culture, the effects of nitrogen amount and the ratio of NO_3^-/NH_4^+ in the medium on cell growth and anthocyanin production were investigated. Total nitrogen amount and the ratio of NO_3^-/NH_4^+ in the medium strongly affected anthocyanin production and cell growth. When NH_4^+ was fixed, the cell growth was promoted by 50 mM total nitrogen (20 mM NO_3^- : 30 mM NH_4^+) than other nitrogen combinations, and was strongly inhibited when NO_3^- was lacking (0 mM NO_3^- : 60 mM NH_4^+) while anthocyanin production was increased. When NO_3^- was fixed, the cell growth was promoted by 70 mM total nitrogen (40 mM NO_3^- : 30 mM NH_4^+) than other nitrogen combinations, and was strongly inhibited when NO_3^- was lacking (0 mM NO_3^- : 60 mM NH_4^+) while anthocyanin production was increased. Cell growth was gradually increased by all nitrogen combinations, but anthocyanin production reached its peak on day 4 in culture. Anthocyanin content increased with decreasing cell density. Sucrose was rapidly hydrolyzed to fructose and glucose within 4 days. Glucose and fructose concentrations in the medium increased and peaked at the 4th day. The anthocyanin content of NH_4^+-free 2% sucrose media was 2 times (200 μg/g) higher than that of 1% sucrose. When NO_3^- was lacking, the highest anthocyanin production was observed at 4% sucrose after 12 days of culture, and increased along with the sucrose concentration.

Introduction

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). Anthocyanin is fundamentally responsible for different color between grapes and the resultant wines (Ribéreau-Gayon, 1982).

Usually, 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: 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 dedifferentiated and grow rapidly in cultures (Ozeki and Komamine, 1981). 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). 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). Anthocyanin production is influenced by different factors

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Received Apr. 14, 2002; accepted May. 20, 2002.

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 high sugar concentration (Do and Cormier, 1990), and as a consequence 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. 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; 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. *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 et al., 1986).

Enhancement of anthocyanin productivity can be affected by many 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).

This study was designed to investigate the effects of nitrogen source on *in vitro* cell growth and pigment production 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 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°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. Longitudinal-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%.

Nitrogen source

Cell suspension cultures were grown in the MS basal medium containing 20 mM NH_4^+, 40 mM NO_3^-, and 2%

sucrose in darkness. To investigate the combined effect of nitrogen source, NH_4^+ (NH_4Cl) and NO_3^- (KNO_3) were combined as follows. When NH_4^+ was fixed to 20 mM, 0, 30, or 50 mM NO_3^- was applied. On the other hand, 0, 10, or 30 mM NH_4^+ was applied in combination with fixed 40 mM NO_3^-.

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 $\times g$ 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 $\mu g/g^{-1}$ 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

Total nitrogen in MS basal medium was 60 mM (potassium nitrate: 40 mM, ammonium nitrate: 20 mM). In MS medium containing 70 mM nitrogen in the form of potassium nitrate and ammonium nitrate, the molar ratio of ammonium ion to nitrate ion was about one to three. To investigate the effect of both types of nitrogen, inorganic nitrogen sources in the medium were prepared with potassium nitrate or ammonium chloride in the equimolar concentration.

Total amount of nitrogen in the medium and the ratio of NO_3^- to NH_4^+ strongly affected anthocyanin production and cell growth. When NH_4^+ was fixed, cell growth did not occur and even decreased on NO_3^--free media. It was thought that cells were killed. The media with varying NO_3^- levels resulted in nearly the same growth, showing sharp increase from day 4 in culture, and slow increase from 12 days in culture (Figure 1).

When NO_3^- was fixed, NH_4^+-free media resulted in growth decrease although suspended the growth of NO_3^--free media (Figure 2). In contrast to cell growth, anthocyanin production was significantly increased when the medium was devoid of NO_3^-, the peak of which reached after 4 days in culture, and slight decrease was observed thereafter (Figure 3). Anthocyanin production was the highest with NH_4^+-free media with the peak at day 4 in culture, and decreased slightly thereafter (Figure 4).

Total anthocyanin content increased throughout the course of the culture for 16 days, showing the maximum with 50 mM NO_3^- and the minimum with NO_3^--free media (Figure 5). Total anthocyanin was the lowest when NH_4^+ was lacking as well as NO_3^-, the rest of the treatments resulted in similar trends, showing gradual increase (Figure 6).

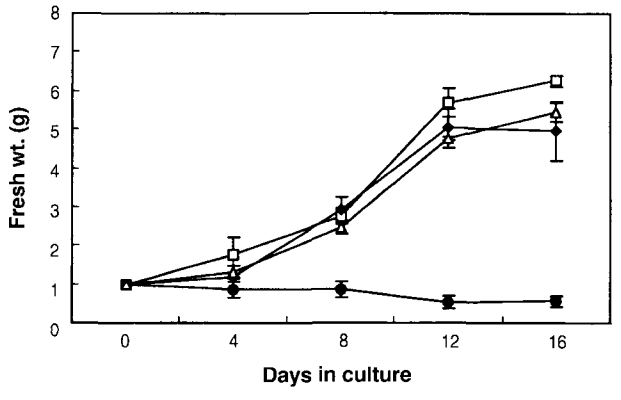

Figure 1. Fresh weight of 'Sheridan' grape cell suspension cultures in MS medium containing 60 mM NH_4^+ (●), and 20 mM NH_4^+ + 30 mM (□), 40 mM (◆ MS basal medium) and 50 mM NO_3^- (△). Bars represent ± SE.

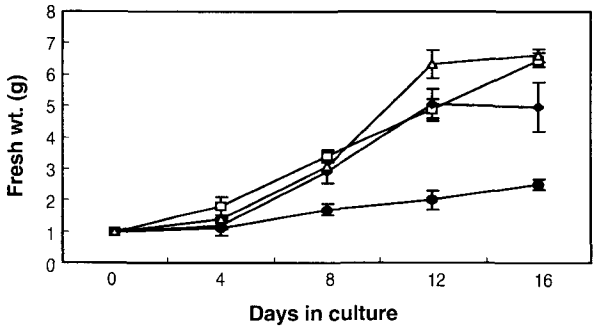

Figure 2. Fresh weight of 'Sheridan' grape cell suspension cultures in MS medium containing 60 mM NO_3^- (●), and 40 mM NO_3^- + 10 mM (□), 20 mM (◆ MS basal medium) and 30 mM NH_4^+ (△). Bars represent ± SE.

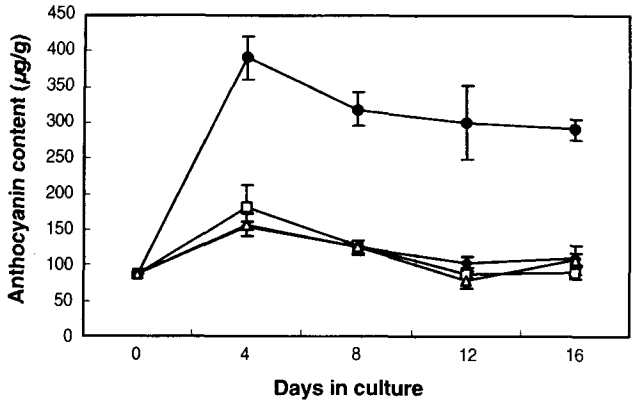

Figure 3. Anthocyanin content of 'Sheridan' grape cell suspension cultures in MS medium containing 60 mM NH_4^+ (●), and 20 mM NH_4^+ + 30 mM (□) , 40 mM (◆ MS basal medium) and 50 mM NO_3^- (△). Bars represent ± SE.

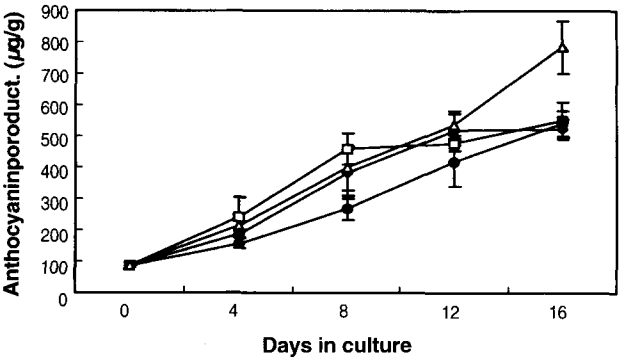

Figure 5. Time course anthocyanin productivity of 'Sheridan' grape cell suspension cultures inoculated in medium containing 60 mM NH_4^+ (●), and 20 mM NH_4^+ + 30 mM (□) , 40 mM (◆ MS basal medium) and 50 mM NO_3^- (△). Bars represent ± SE.

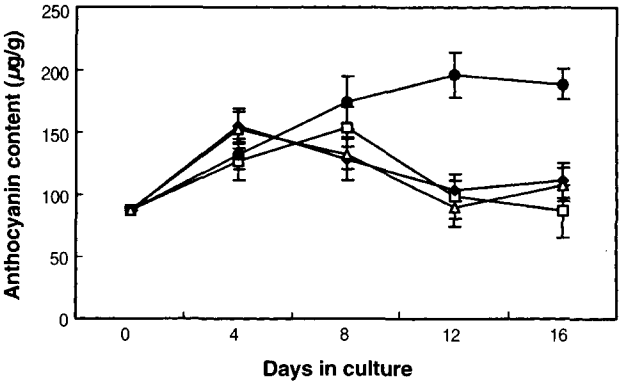

Figure 4. Time course anthocyanin content of 'Sheridan' grape cell suspension cultures inoculated in medium containing 60 mM NO_3^- (●), and 40 mM NO_3^- + 10 mM (□) , 20 mM (◆ MS basal medium) and 30 mM NH_4^+ (△). Bars represent ± SE.

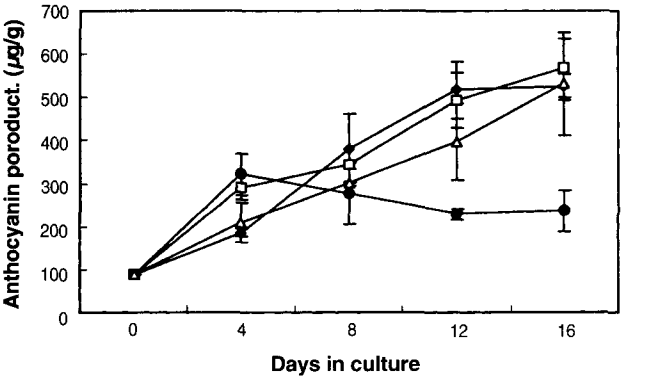

Figure 6. Time course anthocyanin productivity of 'Sheridan' grape cell suspension cultures inoculated in medium containing 60 mM NO_3^- (●), and 40 mM NO_3^- + 10 mM (□) , 20 mM (◆ MS basal medium) and 30 mM NH_4^+ (△). Bars represent ± SE.

When either NH_4^+ or NO_3^- was lacking, cell growth decreased while anthocyanin content was high. When NO_3^- was lacking, cell growth increased slightly and anthocyanin content was relatively low. It was thought that NO_3^- affected cell growth while NH_4^+ affected anthocyanin production. Anthocyanin content was low from 4 days in culture. It was shown that anthocyanin accumulation began when there was no multiplication of cells, and when cell multiplication began, anthocyanin accumulation was diminished.

Extracellular anthocyanin in the medium was not detected at any stage during the course of culture. As pointed out in suspension cultures of *Vitis vinifera*, anthocyanin catabolism is closely related to cell membrane integrity. With cellular lysis, the anthocyanins stored in anthocyanoplasts or vacuoles are released into the culture medium and are quickly metabolized (Guardiola et al., 1995). As a result, extended anthocyanin production could not be sustained due to the loss of cell viability and nutrient depletion. The optimum culture period was found to be 12 days in this study. However, a major limitation for continuous cultures of plant cells is that the cells must release the targeted products extracellularly. In NO_3^- deficiency, culture medium pigmented because anthocyanin came out from cell. All nitrogen combinations were gradually increased the cell growth. But anthocyanin production peaked on day 4 in culture (Figure 2). The high concentration of ammonium ion in the production medium reduced the accumulation of anthocyanin. Several authors have observed the negative effect of ammonium on anthocyanin formation in cell culture. In *Euphorbia millii* cells, anthocyanin production was inhibited by high concentrations of

ammonium ion (Yamamoto et al., 1989). When NO_3^- was fixed, cell growth was promoted by 70 mM total nitrogen (40 mM NO_3^- : 30 mM NH_4^+) than other nitrogen combinations. Cell growth was strongly inhibited when NO_3^- was lacking (0 mM NO_3^- : 60 mM NH_4^+) while anthocyanin production was increased (Figure 3). All nitrogen combinations gradually increased cell growth. But anthocyanin production reached the peak on day 4 in culture (Figure 4). Anthocyanin content increased with decreasing cell density. These phenomena resulted in an increase in the percentage of pigmented cells in a culture. By limiting the nutrients, the metabolic pathway may be stopped and then shifted to the anthocyanin production state from the growth state; the cell cycle has stopped at a period in the cell cycle due to nutrient limitation (Gould et al., 1981). Cell growth and anthocyanin production of nitrogen source were dependent on NH_4^+ and the total nitrogen concentration.

The pH decreased at the early stage (after 4 days culture) and then gradually increased thereafter (Figures 7, 8). The best performance of cell growth was observed in pH 6 ~8 after 12 days in culture, while anthocyanin production showed the maximum in pH 3~5 after 4 days in culture.

Figure 9 and 10 show the dynamic changes of glucose and fructose during culture. It was reported that sucrose was rapidly hydrolyzed to fructose and glucose within 3 to 4 days by extracellular and cell wall-bound invertase (Kanabus et al., 1986; Ueda et al., 1974). Glucose and fruc-

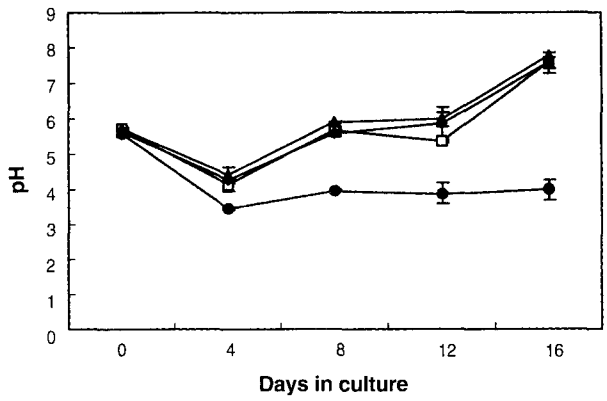

Figure 7. Time course change in medium pH of 'Sheridan' grape cell suspension cultures inoculated in medium containing 60 mM NH_4^+ (●), and 20 mM NH_4^+ + 30 mM (□) , 40 mM (◆ MS basal medium) and 50 mM NO_3^- (△). Bars represent ± SE.

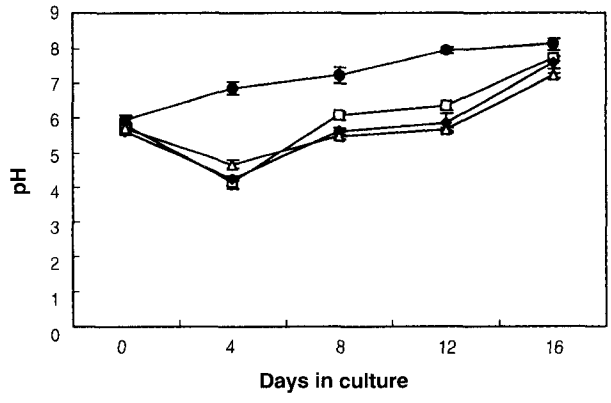

Figure 8. Time course change in medium pH of 'Sheridan' grape cell suspension cultures inoculated in medium containing 60 mM NO_3^- (●), and 40 mM NO_3^- + 10 mM (□) , 20 mM (◆ MS basal medium) and 30 mM NH_4^+ (△). Bars represent ± SE.

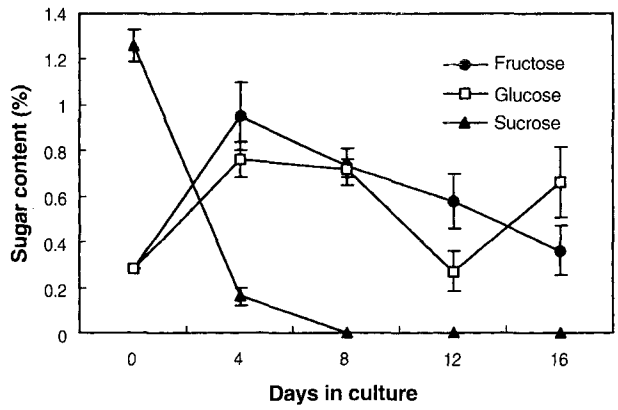

Figure 9. Time course change of sugar content in medium of 'Sheridan' grape cell suspension cultures inoculated in medium containing 0 mM NO_3^- + 60 mM NH_4^+. The vertical bar represent SE.

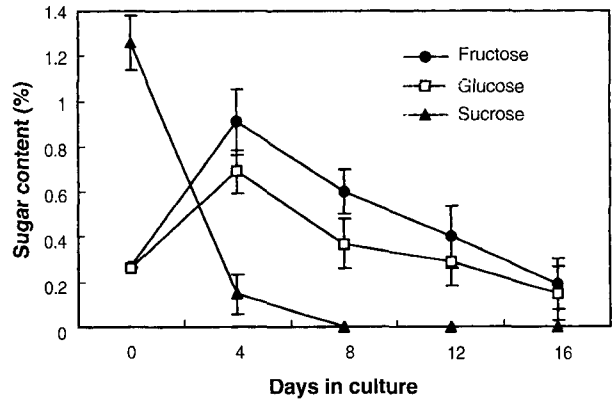

Figure 10. Time course change of sugar content in medium of 'Sheridan' grape cell suspension cultures inoculated in medium containing 0 mM NH_4^+ + 60 mM NO_3^-. The vertical bar represent SE.

tose concentration in the medium increased and peaked at day 4. Pepin et al. (1995) reported that glucose was preferentially consumed before fructose in *Vitis vinifera*. Similar result was observed in this experiment (Figures 8 and 9). However, Hong et al. (1989) reported that all the sucrose added to the culture broth was converted to fructose and glucose within a day after inoculation. This finding may reflect the differences in invertase activity between different cell lines. In this experiment, fructose concentration increased during 16 days of culture when NO_3^- was lacking, because anthocyanin came out from the cell to the medium.

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.

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