Plant Cell, Tissue and Organ Culture **36**: 21–26, 1994.

Anthocyanin production in cultured cells of *Aralia cordata* Thunb.

Kazuo Sakamoto[1], Kumiko Iida[1], Kimiko Sawamura[1], Kyoko Hajiro[1], Yoshihisa Asada[2], Takafumi Yoshikawa[2] & Tsutomu Furuya[2]
[1]*Tonen Corporation, 1–3–1 Nishi-tsurugaoka, Ohi-machi, Iruma-gun, Saitama 354, Japan*; [2]*School of Pharmaceutical Sciences, Kitasato University, 5–9–1 Shirokane, Minato-ku, Tokyo 108, Japan*

Received 15 October 1992; accepted in revised form 16 July 1993

Key words: anthocyanin-producing cell line, *Aralia cordata*, callus culture, small-cell-aggregate selection

Abstract

High anthocyanin-producing cell lines, which were grown in a dark or in a light-dark regime, were selected from callus cultures initiated from stem and leaf tissues of *Aralia cordata* Thunb. by small-cell-aggregate selection. To verify the optimum culture conditions for anthocyanin production, cells were tested by changing the various basal media, sucrose concentration and nitrogen source and concentration. Good growth was obtained in the dark on Linsmaier-Skoog's basal medium containing 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin, 2% (w/v) sucrose and full strength of nitrogen concentration. However, the highest anthocyanin yield (10.3% dry wt) was obtained in the dark on B5 medium containing 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin. Our results suggested that it has became feasible to find the most effective conditions for cell growth and anthocyanin production by optimizations of the nitrogen concentration and the ratio of NH_4^+ to NO_3^- in the medium.

Abbreviations: B5 – Gamborg (Gamborg et al. 1968), 2,4-D – 2,4-dichlorophenoxyacetic acid, LS – Linsmaier and Skoog (Linsmaier & Skoog 1965), MS – Murashige and Skoog (Murashige & Skoog 1962), NN – Nitsch and Nitsch (Nitsch & Nitsch 1967), WH – White (White 1963)

Introduction

Anthocyanins are widely found in various plant species, and are most conspicuous in flower and fruit parts. Because of their low toxicity, anthocyanins have a high potential as a food additive and marker, so many institutes and food manufacturers are engaged in intensive research to produce these pigments from various plant cell cultures: *Euphorbia millii* (Yamamoto et al. 1982), *Callistephus chinensis* (Rau & Forkman 1986), sweet potato (Nozue et al. 1987), *Centaurea cyanus* (Kakegawa et al. 1987), *Hibiscus sabdariffa* (Mizukami et al. 1988), *Perilla frutescens* (Zhong et al. 1991), etc. Anthocyanins, however, usually accumulate only in small amounts in cultured plant cells and their production generally requires light irradiation. Only a few plant cell cultures have been reported to produce anthocyanins in the dark, but the anthocyanin levels are very low: *Ajuga reptans* (Callebaut et al. 1990), *Daucus carota* (Dougall et al. 1980; Kinnersley & Dougall 1980), *Vitis* hybrid (Yamakawa et al. 1983) and *Bupleurum falcatum* (Hiraoka et al. 1986). To date, no reports have appeared on the selection of high anthocyanin-

This paper is part 81 in the series 'Studies on Plant Tissue Cultures'. For Part 80 see Furuya T, Sakamoto K, Iida K, Asada Y, Yoshikawa T, Sakai S & Aimi N (1992) Phytochemistry 31: 3065–3068.

producing cell lines grown in the dark. Therefore, it has been difficult to produce anthocyanin at commercially viable levels.

Aralia cordata Thunb. (Araliaceae), which is called udo in Japan, has long been known as an edible wild plant with a distinctive flavor and fragrance, and its roots are also used in traditional Chinese medicine (as Dokkatsu). It is known that the roots contain several kinds of monoterpenes (Yoshihara & Hirose 1973) or diterpenes (Mihashi et al. 1969) and that six triterpenoid saponins (Kawai et al. 1989) are contained in the aerial parts of the plant. During germination, the sprout tip of *A. cordata* is reddish-purple, but it has not been generally known that the plant produces the anthocyanin pigment in quantity. We found a highly productive cell line by subculturing callus derived from *A. cordata* leaves and stems. Anthocyanin-producing cell strains with a high, stable production capacity were obtained by continuous cell-aggregate cloning. We report here the effects of basal media, sucrose concentration and nitrogen source and concentration on anthocyanin production by these *A. cordata* cell cultures.

Materials and methods

Plant material

Aralia cordata Thunb. calli were obtained from young leaves and stems in May 1985. The explants were surface-sterilized with 70% (v/v) ethanol and 10% (w/v) sodium hypochlorite solution followed by rinsing with sterile distilled water. The explants (1 cm square) were transferred onto 40 ml of MS agar medium (0.9% w/v, pH 5.8) supplemented with 1.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} kinetin, 3% sucrose (designated DK medium) in 100 ml Erlenmeyer flasks, and then incubated for 3–4 weeks at 25°C in the dark. The calli were maintained by periodic transfer to fresh media in the dark and/or light-dark cycle (16h–8h). Two anthocyanin-producing cell lines, a dark grown line and a light grown line, were isolated from each original callus culture by selectively subculturing the cell cluster which visually contained anthocyanin pigment. The selection procedure using small cell aggregates was carried out every 3 weeks under the same conditions. After cloning, both anthocyanin-producing cell lines were subcultured every 3 weeks.

Examination of optimal culture medium

The culture conditions were as follows: MS, LS, WH, NN or B5 were used as the basal inorganic and vitamin medium with 0.2% (w/v) gellan gum in a Petri dish (6.0 cm in diameter). Sucrose at 1, 2, 3, 4, 5, 6, 9 or 12 % (w/v) was further added to MS, LS or B5 basal medium containing 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin. The nitrogen concentration was caused by adding 0, 1/5, 1/3, 1/2, 1/1 or twice the total nitrogen amount of each original basal medium. Calli of *A. cordata* (1g fresh weight) growing on DK medium were transferred to the various media as above. These calli were maintained at 25°C in the dark and/or light-dark cycle (16h–8h, 3,000–5,000 lux) for 3 weeks. All experiments were done in three replicates.

Measurement of growth and determination of anthocyanin content

Callus growth was measured by determining fresh and dry weight (freeze dried, 7 days). Dried cells (10 mg) were extracted overnight in 0.1% (v/v) HCl-methanol (10 ml) at 4°C. After centrifugation at 2,000 rpm for 10 min, 1 ml of the clean supernatant was diluted 3-fold with the same acidic methanol solution. The absorbance of the methanolic solution was measured at 530 nm, which was the λmax of this anthocyanin solution, with a spectrophotometer. The anthocyanin content was estimated from a working curve which was generated by a cyanin chloride standard and the yield (%/dry cell weight (g)) was calculated.

Results and discussion

Selection of anthocyanin producing cell lines

Six weeks after culturing explants of young leaves and stems of *A. cordata* on DK medium in the dark, we obtained dark-brown and creamy

colored spotted calli. These calli were also transferred to a light (16h)-dark (8h) regime. After several subcultures, a friable faster growing callus was obtained for each treatment and small red colored spots appeared in these calli. By continuous cell-aggregate selection, cell lines having a fast growth rate and increased anthocyanin production were established in callus cultures grown in the dark and in the light. These cell lines, which contained anthocyanin, were established 4 to 5 months after the beginning of selective subculturing.

Growth and production of anthocyanins in callus cultures

The growth and anthocyanin production curves of high producing cell lines in the dark or under a light-dark regime are illustrated in Fig. 1. From day 6 after transfer to fresh medium, the fresh weight of cells grown in darkness rapidly increased as compared to the fresh weight of light grown cultures. The growth of both cell lines attained a maximum after four weeks. Anthocyanin biosynthesis of these cells continued during all growth stages, anthocyanin accumulated from the initial transfer to fresh medium and reached a maximum after three weeks. The maximum anthocyanin yield of the calli grown on DK medium in the dark fluctuated between 7 and 8% (dry wt), and in the light around 6% during the experiment period of 7 years (Fig. 2). Even after long-term subcultures, the ability of the cell lines to produce a high amount of anthocyanin has been maintained. It was usually observed that on DK medium, the anthocyanin content of the dark grown calli was higher than that of the light grown cultures. This suggests that anthocyanin biosynthesis of *A. cordata* cell cultures is not entirely dependent on light irradiation.

From these dark or light grown cultures, suspension cultures could be established for each treatment. For the suspension cultures, the productivity was maintained, and the growth period to reach the stationary phase was reduced to one week by comparison with the static callus cultures (data not shown) as a result of their rapid growth. When the culture period of suspension cultures was greater than 2 to 3 weeks, cell-browning occurred by the formation of polyphenolics, causing cell death. Whereas the cell suspension cultures obtained from *B. falcatum* (Hiraoka et al. 1986) and *A. reptans* (Callebaut et al. 1990) were heterogeneous, it was confirmed by microscopic observations that the

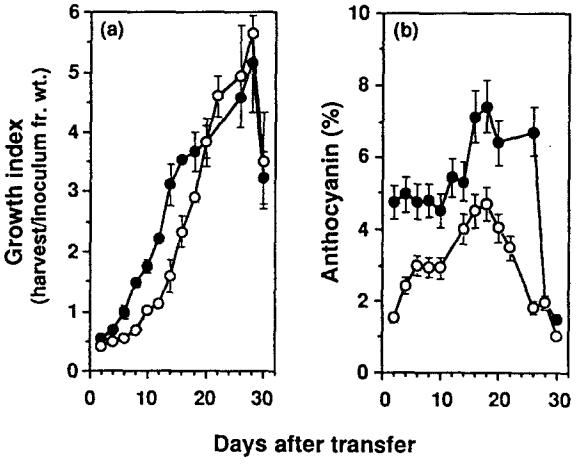

Fig. 1. (*a*) Growth and (*b*) anthocyanin production in callus cultures of *A. cordata* grown in darkness (solid circles) and in a light-dark regime (open circles). Cultures were maintained in MS medium with 3% (w/w) sucrose, 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin at 25°C. Vertical lines show standard errors for three replicates.

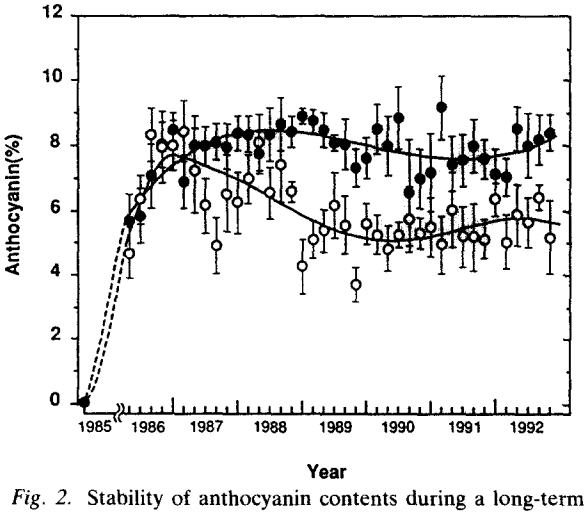

Fig. 2. Stability of anthocyanin contents during a long-term subculturing of *A. cordata* callus grown in darkness (solid circles) and in a light-dark regime (open circles). Cultures were maintained in MS medium with 3% (w/w) sucrose, 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin at 25°C for 21 days. Vertical lines show standard errors for five replicates.

anthocyanin-producing cells in *A. cordata* cell suspension cultures were highly homogeneous (anthocyanin-producing cells/total cells ≧0.90).

Effects of basal inorganic media

The effect of several basal inorganic media on growth and anthocyanin production of callus cultures in the dark or under the light conditions is shown in Fig. 3. Growth in the dark was similar on MS, B5 and NN, however lower than in LS medium. The growth rate in LS medium was 1.6-times higher than that in DK medium. Only WH medium gave poor growth. Anthocyanin production in the dark was similar to that with the DK medium used for repetitive subculturing, except for WH medium. The highest anthocyanin yield (9.4%) was obtained on B5 medium. Under a light-dark regime, B5 medium gave a relatively higher growth rate. LS, MS and NN medium were similar to each other. The anthocyanin yield on B5 medium, on the contrary, was lower than that on LS, MS and NN media. A similar effect was shown in *Euphorbia millii* (Yamamoto et al. 1989) cell suspension cultures. WH medium, whihc is poor in mineral salts as compared to the other basal medium, gave poor growth and anthocyanin yield in the dark and light. Growth and anthocyanin production data on basal inorganic medium in the dark or under a light-dark cycle showed that the highest biomass and yields were obtained in the dark rather than under the light condition.

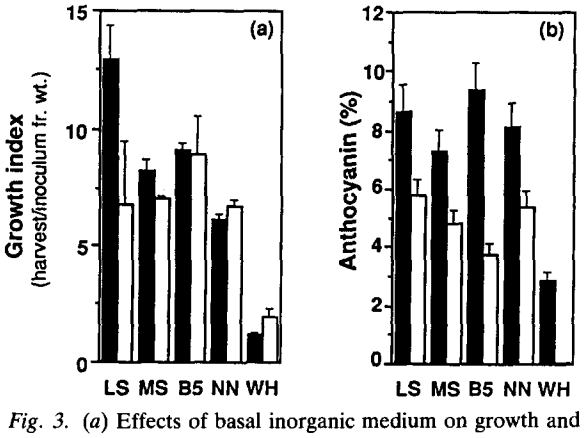

Fig. 3. (*a*) Effects of basal inorganic medium on growth and (*b*) anthocyanin production in callus cultures of *A. cordata* grown in darkness (solid bars) and in a light-dark regime (open bars). Cultures were maintained in various basal medium with 3% (w/w) sucrose, 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin at 25°C for 21 days. Vertical lines show standard errors for three replicates.

Influence of sucrose concentration

The effect of sucrose concentration on cell growth and anthocyanin production in the inorganic basal medium of LS, MS and B5 is shown in Fig. 4. In both dark and light conditions, cell growth was reduced in excess of 5% (w/v) sucrose concentration. Higher sucrose concentrations (9 or 12%) remarkably reduced growth on all basal media. This is probably caused by the higher osmotic strength of the media. It has been confirmed by microscopic observation that the anthocyanins accumulated in the vacuoles of the cultured cells of *A. cordata*. The higher osmotic strength probably negatively affected the water content of the vacuole, so the anthocyanin

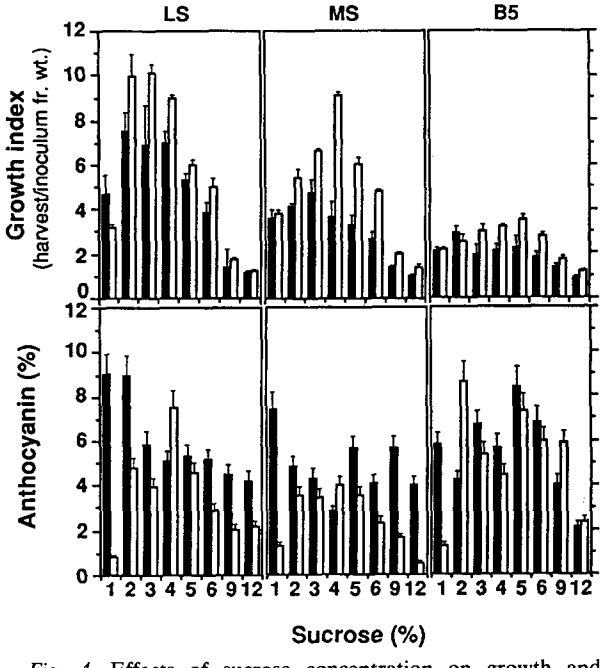

Fig. 4. Effects of sucrose concentration on growth and anthocyanin production in callus cultures of *A. cordata* grown in darkness (solid bars) and in a light-dark regime (open bars). Cultures were maintained in LS, MS and B5 basal medium with 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin at 25°C for 21 days. Vertical lines show standard errors for three replicates.

accumulation was limited by the higher sucrose concentration. *A. reptans* (Callebaut et al. 1990) and *Catharanthus roseus* callus (Carew & Krueger 1976) also tended to show reduced growth and production at higher sucrose concentrations. The highest growth was obtained on LS basal medium with 2 to 4% sucrose concentration in the dark and light. The highest anthocyanin production was observed on LS basal medium with 2% sucrose concentration in the dark and on B5 basal medium with 2% sucrose concentration in the light, respectively. The best conditions for total anthocyanin production in the dark and light were on LS basal medium with 2% sucrose concentration (anthocyanin yield; 9.0%, growth index; 7.5) and 4% sucrose concentration (anthocyanin yield; 7.5%, growth index; 8.9), respectively.

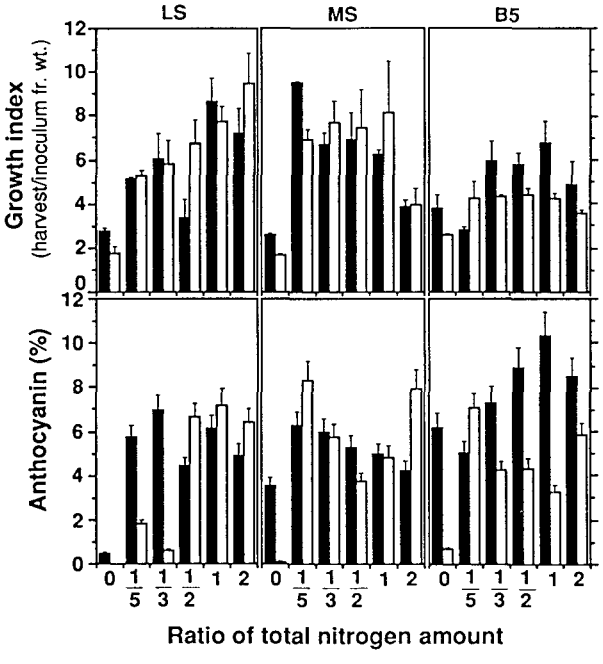

Fig. 5. Effects of total nitrogen amount on growth and anthocyanin production in callus cultures of *A. cordata* grown in darkness (solid bars) and in a light-dark regime (open bars). The total nitrogen amount is expressed as the fraction of the total nitrogen concentration in each basal medium. Cultures were maintained in LS, MS and B5 basal medium with 3% (w/w) sucrose, 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin at 25°C for 21 days. Vertical lines show standard errors for three replicates.

Influence of nitrogen amount and nitrogen source

Figure 5 shows the total amount of nitrogen (NO_3^- and NH_4^-) in the basal media affected growth and anthocyanin production. In the dark, cell growth was better promoted by 1/5 of the total nitrogen of the standard MS medium. On the MS medium, the anthocyanin yield was the highest with the same nitrogen concentration and decreased gradually with increasing nitrogen concentration. On the other hand, the highest anthocyanin yield (10.3% dry wt.) was obtained in the B5 medium and decreased gradually with decreasing nitrogen concentration. The amount of the NH_4^+ ion in the B5 medium is significantly less than that of LS or MS medium. This suggests that the anthocyanin biosynthesis in the dark was activated by the presence of the NO_3^- ion alone. From the investigation of the effects of the ratio of NH_4^+ to NO_3^- in the MS medium on anthocyanin production, it has also been demonstrated that a higher ratio of NO_3^-/NH_4^+ was effective for anthocyanin production in the dark and light (data not shown). In the light, the effect on anthocyanin yield obtained on B5 medium varies inversely to the situation in the dark, and the yield was higher at a 1/5 nitrogen concentration of the standard B5 medium.

Further investigations are in progress to characterize the main anthocyanin component in the cultured cells of *A. cordata*, and attempts to improve the anthocyanin productivity are also being made.

References

Callebaut A, Hendrickx G, Voets AM & Motte JC (1990) Anthocyanins in cell cultures of *Ajuga reptans*. Phytochemistry 29: 2153–2158

Carew DP & Krueger RJ (1976) Anthocyanidins of *Catharanthus roseus* callus cultures. Phytochemistry 15: 442

Dougall DK, Johnson JM & Whitten GH (1980) A clonal analysis of anthocyanin accumulation by cell cultures of wild carrot. Planta 149: 292–297

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

Hiraoka N, Kodama T & Tomita Y (1986) Selection of

Bupleurum falcatum callus line producing anthocyanins in darkness. J. Nat. Prod. 49: 470–474

Kakegawa K, Kaneko Y, Hattori E, Koike K & Takeda K (1987) Cell cultures of *Centaurea cyanus* produce malonated anthocyanin in uv light. Phytochemistry 26: 2261–2263

Kawai H, Nishida M, Tashiro Y, Kuroyanagi M, Ueno A & Satake M (1989) Studies on the structures of udosaponins A, B, C, D, E and F from *Aralia cordata* Thunb. Chem. Pharm. Bull. 37: 2318–2321

Kinnersley AM & Dougall DK (1980) Increase in anthocyanin yield from wild-carrot cell cultures by a selection system based on cell-aggregate size. Planta 149: 200–204

Linsmaier EM & Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100–127

Mihashi S, Yanagisawa I, Tanaka O & Shibata S (1969) Further study on the diterpenes of *Aralia* spp. Tetrahedron Lett. 21: 1683–1686

Mizukami H, Tomita K, Ohashi H & Hiraoka N (1988) Anthocyanin production in callus cultures of roselle (*Hibiscus sabdariffa* L.). Plant Cell Rep. 7: 553–556

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Nitsch C & Nitsch JP (1967) The induction of flowering in vitro in stem segments of *Plumbago indica* L. I. The production of vegetative buds. Planta 72: 355–370

Nozue M, Kawai J & Yoshitama K (1987) Selection of a high anthocyanin-producing cell line of sweet potato cell cultures and identification of pigments. J. Plant Physiol. 129: 81–88

Rau D & Forkman G (1986) Anthocyanin synthesis in tissue cultures of *Callistephus chinensis* (China aster). Plant Cell Rep. 5: 435–438

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

Yamamoto Y, Mizuguchi R & Yamada Y (1982) Selection of a high and stable pigmentproducing strain in cultured *Euphorbia millii* cells. Theor. Appl. Genet. 61: 113–116

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

Yoshihara K & Hirose Y (1973) Terpenes from *Aralia* species. Phytochemistry 12: 468

White PR (Ed) (1963) The Cultivation of Animal and Plant Cells (p. 228). The Ronald Press, New York

Zhong J, Seki T, Kinoshita S & Yoshida T (1991) Effect of light irradiation on anthocyanin production by suspended culture of *Perilla frutescens*. Biotechnol. Bioeng. 38: 653–658