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Original Paper

Production of phytochemicals by using habituated and long-term cultured cells

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Abstract Cultured plant cell lines that grew and produced phytochemicals in the absence of exogenous plant hormones were established and then examined for their culture characteristics and the features of their produced phytochemicals. Cell lines that produce the following phytochemicals were established by selection and passage: anthocyanin-producing cell lines of cherry, peach, and Asiatic dayflower (*Commelina communis* L.); a carotenoid-producing cell line of Asiatic dayflower; a betacyanin-producing cell line of an *Amaranthus* species; and an isoflavone-producing cell line of soybean. The strains of cherry, peach, and Asiatic dayflower were established by passage in hormone-free media, whereas those of the *Amaranthus* sp. and soybean required passage with concentrations of auxins and cytokinins that were progressively halved. The anthocyanin-producing cell line of pear required low levels of phytohormones ($0.156\,\mu g\,l^{-1}$ of 2,4-dichlorophenoxyacetic acid and $0.0156\,\mu g\,l^{-1}$ of 6-benzylaminopurine), and no cell line that grew in the absence of exogenous phytohormones could be obtained from pear. The anthocyanins malonylawobanin and flavocommelin of blue Asiatic dayflower petal were not found in extracts from the blue-violet anthocyanin-producing cultured cells. The anthocyanin-producing cherry cell line that requires no phytohormone is amenable to long-term culture over 300 passages. In suspension culture, the strain produces anthocyanin at a higher concentration in modified B5 medium than in Murashige and Skoog medium.

Key words: Cell culture, habituation, phytochemical, phytohormone.

To produce phytochemicals by plant cell culture, it is necessary to control the secondary metabolic system as outlined by Payne et al. (1991) and Fu et al. (1999). The production of anthocyanins, in particular, has been examined (Deroles 2009). Anthocyanins are flavonoid pigments found in plants. Because anthocyanins are widely used in foods, pharmaceuticals, cosmetics, and → pigments, it is necessary to remove synthetic phytohormones, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthylacetic acid (NAA), 6-benzylaminopurine (BA), or kinetin, from the culture medium during process of extracting and purifying the products. This purification step would be unnecessary if it were possible to establish cultured cell lines that do not require the presence of phytohormones for growth and the production of phytochemicals.

Several studies on cultured cells that do not require the addition of phytohormones have been reported, for example, for *Lilium* (Sheridan 1968), tobacco (Binns and Meins 1973; Syono and Furuya 1974), sugarbeet (Kevers et al. 1981), and soybean (Wyndaele et al. 1988). In addition, indol-3-acetic acid has been detected as an endogenous phytohormone in cells of cell lines that do not require exogenous phytohormone (Nishio et al. 1976; Wyndaele et al. 1988), and these cells have been subjected to physiological analyses. An anthocyanin-producing cell line of strawberry (Asano et al. 2002) is a rare exception that requires no exogenous phytohormones and produces phytochemicals at a rate of over $10\,mg\,g^{-1}$ (1%) dry weight.

Plant cells present a problem in that cell lines die out unless they are stored in a freezer or they are subcultured. Although studies on embryogenic cells (Maruyama et al. 2000) and suspension cultures (Kobayashi et al. 2005; Sung et al. 2000) have been attempted, not all cultured cells can be stored in a freezer. Moreover, long-term continuous subculture to maintain a cell culture is necessary for industrial applications.

In this study, we were successful in establishing cell

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzylaminopurine; HPLC, high-performance liquid chromatography; MB5 medium, modified Gamborg's B5 medium; NAA, 1-naphthylacetic acid; TFA, trifluoroacetic acid.

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lines from a number of species, and some of these cell lines were found to require no exogenous phytohormones. Among the substances that can be produced by plant cell lines, anthocyanins of cherry, peach, strawberry, and pear are perceived as being safe for use as food colorants. We also established cell lines of the edible Asiatic dayflower (Yamada 1992) that produce a blue anthocyanin; of an edible *Amaranthus* sp. (Makino 1977) that contains betacyanin, potentially useful as a food colorant and antioxidant; and of soybean, which produces isoflavones, which are well-known functional materials. All these products are expected to have functional value.

The culture characteristics of the cell lines and the features of the phytochemicals that they produced were examined, and the possibilities were assessed of long-term subculture and large-scale suspension culture of phytochemical-producing cell lines that require no exogenous phytohormones. We also examined the suspension culture and growth characteristics of anthocyanin-producing cherry cells in long-term subculture without phytohormones to verify that such a large-scale culture system is viable at a practical level.

Materials and methods

Culture media

For this study, we used Murashige and Skoog's (1962) (MS) medium and Gamborg's modified B5 (MB5) medium comprising the inorganic salts of the B5 medium (Gamborg et al. 1968) and the organic components of the MS medium. The sucrose content of the media was 3% (w/v) and their pH was adjusted to 5.7. These media were used in combination with various concentrations of 2,4-D and NAA as auxins, and BA and kinetin as cytokinins. The solid medium that we used contained 0.25% (w/v) of gellan gum (Wako Pure Chemical Industries Ltd., Osaka) as a gelling agent. A 20-ml portion of medium was used in a 100-ml culture flask.

Plant materials and callus culture

Calli induced from organs and tissues were incubated several times during a four-week period; subsequent incubation periods were two to three weeks and then uniformly two weeks once a phytochemical-producing cell line had been established.

Cherry petals and filaments were collected from buds of the cherry *Prunus* × *yedoensis* Matsum. cv. Someiyoshino, one week before flowering. The tissue samples were placed on MS medium supplemented with 2,4-D ($1.0\,mg\,l^{-1}$) and BA ($0.1\,mg\,l^{-1}$) at 25°C in darkness to induce calli. Each callus was subcultured on the same medium under fluorescent lighting (FL40SS·ECW/37; Panasonic Inc., Japan) ($27\,\mu mol\,m^{-2}\,s^{-1}$). The emergent red cells were visually selected to establish red-anthocyanin-producing cell lines. The light intensity was measured by using a photon sensor (Koito IKS-271/101; Koito Industries, Ltd., Japan). Anthocyanin-producing cell lines derived from petals were incubated and maintained on the same medium, whereas those from filaments were incubated and maintained on a medium supplemented with 2,4-D ($0.1\,mg\,l^{-1}$) and BA ($0.1\,mg\,l^{-1}$).

In the case of peach, a tissue section measuring about 5×7×5 mm was removed from a sterilized immature fruit (about 3 cm in diameter) of *Prunus persica* Batsch cv. Yamanehakutou, and formation of calli was induced on MS medium supplemented with 2,4-D ($1.0\,mg\,l^{-1}$) and BA ($0.1\,mg\,l^{-1}$). Thereafter, subculture was continued under light conditions on MS medium supplemented with NAA ($2.0\,mg\,l^{-1}$) and BA ($0.2\,mg\,l^{-1}$) for stable growth without browning. A hard red callus and a soft loose callus were obtained from this line. The hard callus failed to grow and turned brown without producing any anthocyanin-producing cell line. The soft loose callus, on the other hand, grew and was used to establish an anthocyanin-producing cell line.

A cell line of strawberry that required no phytohormones was previously obtained from young leaves of the strawberry *Fragaria ananassa* Duch. cv. Nyoho and subcultured on MB5 medium without the addition of supplementary phytohormones (Asano et al. 2002).

In the case of pear, a tissue section measuring about 5×7×5 mm was removed from a disinfected immature fruit (about 3 cm in diameter) of the pear *Pyrus communis* L. cv. Le Lectier. Formation of calli was induced on MS medium supplemented with 2,4-D ($1.0\,mg\,l^{-1}$) and BA ($0.1\,mg\,l^{-1}$). Subsequent subculture was performed on MS medium supplemented with 2,4-D ($1.0\,mg\,l^{-1}$) and BA ($0.1\,mg\,l^{-1}$), for stable growth, without browning, in the presence of light. A red friable callus and a soft, red, loose callus were obtained from this line. The latter was subcultured and selected to establish an anthocyanin-producing cell line.

Seeds of *Tuyukusa*, the Asiatic dayflower *Commelina communis* L., were disinfected. The seed coat of the germ was removed with a sharp knife and the seeds were sprouted on MS medium. From a section (*ca.* 10 mm) of the hypocotyl of a seedling grown to 7–10 cm, calli were induced on MS medium supplemented with 2,4-D ($1.0\,mg\,l^{-1}$) and kinetin ($1.0\,mg\,l^{-1}$). When subculture was continued on MS medium supplemented with NAA ($2.0\,mg\,l^{-1}$) and BA ($0.2\,mg\,l^{-1}$), the cells became yellow and grew in a stable manner without browning. A strain in which red anthocyanin-producing cells were scattered in the yellow callus was obtained under light conditions, and an orange carotenoid-producing cell line and a blue-violet anthocyanin-producing cell line were established by selection from this strain.

Calli of the *Amaranthus* sp. *Akabiyu* were induced from a section of a disinfected red young leaf *Amaranthus inamoenus*, measuring about 5 × 7 mm, under light conditions on MS medium supplemented with 2,4-D ($0.1\,mg\,l^{-1}$) and BA ($1.0\,mg\,l^{-1}$). Although the resulting calli did not produce betacyanin, they were subcultured on MS medium supplemented with 2,4-D ($0.5\,mg\,l^{-1}$) and BA ($0.2\,mg\,l^{-1}$) to establish a red betacyanin-producing cell line.

Calli of soybean were induced from a hypocotyl in a seed of soybean *Glycine max* Merrill cv. Tanbaguro under light conditions. A white callus was obtained on MS medium supplemented with 2,4-D ($1.0\,mg\,l^{-1}$) and BA ($0.1\,mg\,l^{-1}$), and a yellow-green callus was obtained on MS medium supplemented with NAA ($2.0\,mg\,l^{-1}$) and BA ($0.2\,mg\,l^{-1}$). Both calli were subsequently subcultured on MS medium supplemented with NAA ($2.0\,mg\,l^{-1}$) and BA ($0.2\,mg\,l^{-1}$),

which engendered less browning of cells.

Habituation of the cultured cells

Various methods have been used to establish cell lines that require no hormones. For example, a maize cell line has been established by growth in a medium with a hormone concentration of one one-hundred-sixtieth (Hawes et al. 1985), and a soybean cell line has been established through treatment with 2,4-dinitrophenol and phenoxyisobutyric acid, which have antiauxin activity (Christou 1988). One approach that we used was to halve the hormone concentration in the medium at each passage; this is similar to the method used by Ikeda et al. (1979) to establish a cell line of soybean that required no thiamine, and which we previously used to establish an anthocyanin-producing cell line strawberry that required no hormones (Asano et al. 2002).

Cells that produced phytochemical when grown on the solid medium were incubated on a phytohormone-free medium. This incubation was repeated, after selecting cells that grew and produced phytochemicals, to generate a cell line that was capable of growth and phytochemical production. If growing cells were unobtainable because of browning and death of the cells, we adapted our method as follows. Growing cells that produced phytochemicals were selected on a medium in which the phytohormone concentration was reduced to a half or a quarter of the original concentration, and subculture was repeated using media with this phytohormone concentration until sufficient growth potential and phytochemical productivity were achieved. Selected cells were then grown on a medium with a phytohormone concentration that was again reduced by a half. This process was repeated until a cell line was obtained that was capable of growth and phytochemical production on phytohormone-free medium.

Cell suspension cultures

A 10-g portion of wet cells derived by culture from cherry filaments on the solid medium were subjected to suspension culture in a 250-ml Erlenmeyer flask containing 40 ml of phytohormone-free MS or MB5 liquid medium, and the flask was mounted on a rotary shaker (110 rpm) under lighting conditions that were identical to those used for the solid culture. After two weeks, a 10-ml portion of the resulting suspension culture containing cells was added to 50 ml of fresh medium, and culture was continued. This process was repeated at fortnightly intervals. For suspension culture in the MB5 medium, culture was always maintained in three or more Erlenmeyer flasks, and subculture was continued by transfer to the next medium from the flask that had the highest apparent anthocyanin content (strongest color) by visual inspection.

The average doubling time (D_t) of cherry cells subjected to suspension culture for 14 days was calculated by using the following equations:

$$W_s \times 2^{14/D_t} = W_e \quad (1)$$

$$D_t = 14 \times \log 2 \div \log(W_e/W_s) \quad (2)$$

where W_s is weight of dry cells at the start of the culture, and W_e is the dry weight of cultured cell after 14 days.

Estimation of total phytochemicals

Calli cultured on the basic solid medium were collected and cells cultured in the liquid medium were suction filtered and lyophilized to produce samples suitable for analysis. Anthocyanins were extracted from the dried cells with 0.1% (v/v) HCl–80% (v/v) methanol overnight in a cool dark place. The extract was diluted with 0.1% (v/v) HCl–methanol, and the absorbance was measured at 530 nm using a spectrophotometer (U-1500, Hitachi Ltd., Tokyo). The anthocyanin content was calculated approximately as cyanidin 3-*O*-β-D-glucoside chloride (cyanidin glucoside) by using a value of 29,600 as the molecular extinction coefficient (Wada and Ou 2002). The anthocyanins from the cell culture of Asiatic dayflower and from fresh blue petals of the plant were extracted by using 80% (v/v) methanol and analyzed by high-performance liquid chromatography (HPLC).

Betacyanin was extracted from dried cells of the *Amaranthus* sp. with 50% (v/v) aqueous ethanol and diluted immediately with the same solvent. The absorbance was measured at 530 nm. The content was calculated approximately as amaranthine by using 56,600 as the molecular extinction coefficient at 538 nm, and a molecular weight of 726 (Piattelli et al. 1969).

Isoflavones was extracted from dried cells of soybean with 80% (v/v) methanol, then diluted with an equal volume of 4 M HCl–methanol. The mixture was then heated at 100°C for 60 min to hydrolyze the isoflavones to daidzein and genistein, which were analyzed by HPLC. The sum of both components was recorded as the total isoflavone in milligrams per gram of dry weight.

Carotenoids were extracted from dried cells of the *Amaranthus* sp. with 30% (v/v) methanol–70% (v/v) acetone, and analyzed by HPLC. The total carotenoid content was taken as the sum of the lycopene and β-carotene contents in micrograms per gram dry weight.

Analytical HPLC of phytochemicals

The extracted anthocyanins and betacyanin, and the isoflavones obtained by hydrolysis, were analyzed by HPLC under the following conditions: HPLC system: LC-10 (Shimadzu Corp., Kyoto); photodiode array detector: SPD-M10A (Shimadzu Corp., Kyoto); column: Wakosil-II5C18AR, 4.6×(250+50) mm (Wako Pure Chemical Industries Ltd., Osaka); eluent A: TFA–H_2O (0.5 : 99.5 v/v); eluent B: TFA–AcOH–MeCN–H_2O (0.5 : 40 : 50 : 9.5 v/v); flow rate: 1 ml min^{-1}, column temperature: 40°C; sample volume: 10 μl; gradient: B 5–5% (0–5 min), 5–32.5% (5–60 min).

Anthocyanin and betacyanin were detected at 530 nm, and isoflavone aglycons were detected at 280 nm. Cyanidin glucoside, cyanidin 3-*O*-rutinoside, daidzein, and genistein (Extrasynthese S. A., France) were used as reference substances for confirmation of the retention time, peak spectrum, and quantification.

Extracted carotenoids were analyzed by using the same HPLC system and detector under the following conditions: column: Wakosil-II5C18HG, 4.6×(250+50) mm (Wako Pure Chemical Industries Ltd., Osaka); Eluent A: MeOH; B: EtOAc; flow rate: 1 ml min^{-1}; column temperature: 35°C, sample volume: 5 μl; gradient: B 5–25% (0–40 min), 100% (40–50 min). Carotenoids were detected at 450 nm. Lycopene and β-carotene (Wako Pure Chemical Industries Ltd., Osaka) were

used as reference substances for confirmation of the retention time, peak spectrum, and quantification.

In addition, components were extracted for HPLC analysis from lyophilized cherry petals, filaments, and fruits; from peach fruits; from Asiatic dayflower petals; from leaves of the *Amaranthus* sp.; and from pear anthers. The retention times of the component peaks and the peak spectra were checked in each case.

Results

Establishment and subculture of cell lines requiring no phytohormones

Anthocyanin-producing cell lines derived from cherry petals and filaments were incubated directly on phytohormone-free medium, and cells capable of growth were selected to establish cell lines that required no exogenous phytohormone (Table 1, Figure 1A, B).

By culture on phytohormone-free medium containing sucrose or sorbitol as a carbon source, two anthocyanin-producing cell lines that required no phytohormones were established from peach fruits (Table 1, Figure 1C, D).

To establish an anthocyanin-producing cell line from pear fruit, the phytohormone concentrations were progressively reduced, in 210 steps, from one quarter of their original levels before habituation began to final levels of 0.156 $\mu g\,l^{-1}$ of 2,4-D and 0.0156 $\mu g\,l^{-1}$ of BA, but no cell line that grew in the complete absence of exogenous phytohormone could be obtained (Table 1, Figure 1K). No significant cell browning or death occurred during an initial attempt at culture in a phytohormone-free medium, and anthocyanin was produced, although growth was slow. However, during the second culture on phytohormone-free medium, little if any cell growth was observed and about half the cells turned brown. In the third culture on phytohormone-free medium, no cells showed any red coloration and all the cells died.

Both the blue-violet anthocyanin-producing cell line and the orange carotenoid-producing cell line from the hypocotyl of Asiatic dayflower, on culture on a phytohormone-free medium, directly established cell lines that grew in the absence of phytohormone (Table 1, Figure 1F, G).

The betacyanin-producing cell line from the leaf of the *Amaranthus* sp. was unable to grow on phytohormone-free medium, resulting in browning and death. However, growth potential and the betacyanin-production capacity were retained if the phytohormone concentration in the medium was reduced by a half and culture was performed four times. The procedure of incubating at a phytohormone concentration that was reduced by a half was repeated, and culture was conducted four times at one-eighth of the original phytohormone concentration (0.0625 $mg\,l^{-1}$ 2,4-D, 0.025 $mg\,l^{-1}$ BA). At this stage, the

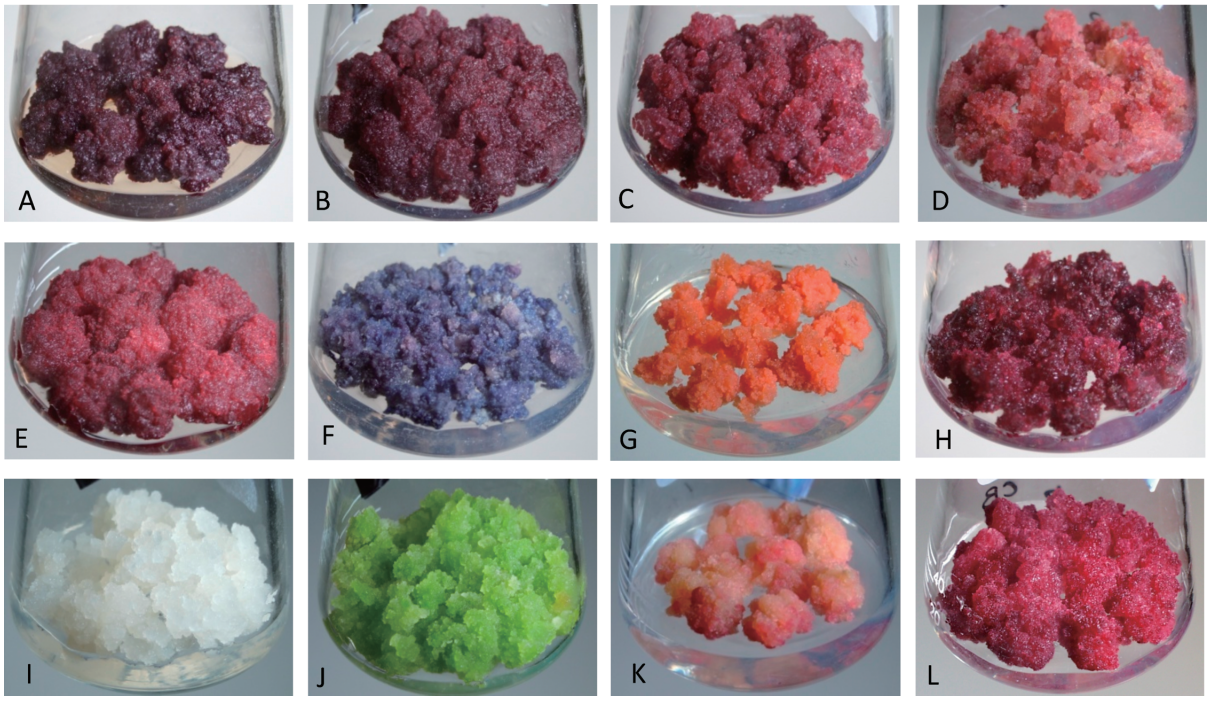

Figure 1. Cultured cells producing phytochemicals. (A) cherry (origin, petal), (B) cherry (origin, filament), (C) peach (carbon source, sucrose), (D) peach (carbon source, sorbitol), (E) strawberry, (F) Asiatic dayflower (phytochemical, anthocyanin), (G) Asiatic dayflower (phytochemical, carotenoid), (H) *Amaranthus* sp., (I) soybean (color, white), (J) soybean (color, yellow-green), (K) pear (phytohormone in medium; 2,4-D 0.156 $\mu g\,l^{-1}$, BA 0.0156 $\mu g\,l^{-1}$), (L) pear (phytohormone in medium; 2,4-D 1.0 $mg\,l^{-1}$, BA 0.1 $mg\,l^{-1}$). (A)–(J) were phytohormone-nonrequiring cell lines.

Table 1. Characteristics of habituated and non-habituated cell lines

Plant		Cherry *Prunus yedoensis* Matsum. cv. Someiyoshino		Peach *Purunus persica* Batsch cv. Yamanehakutou		Strawberry *Fragaria ananassa* Duch. cv. Nyoho	Pear *Pyrus communis* L. cv. Le Lectier	Asiatic dayflower *Commelina communis* L. 'Tsuyukusa'*		Amaranthus sp. *Amaranthus inamoenus* 'Akabiyu'*	Soybean *Glycine max* L. Merrill cv. Tanbaguro	
Outline of cell lines												
Origin organ		Petal	Filament	Fruit		Leaf	Fruit	Hypocotyl		Leaf	Hypocotyl	
Phytochemical		Anthocyanin Cyanidin glycoside		Anthocyanin Cyanidin glycoside		Anthocyanin Cyanidin glycoside Peonidin glycoside	Anthocyanin Cyanidin glycoside	Anthocyanin Delphinidin glycoside	Carotenoid Lycopene β-carotene	Betacyanin	Isoflavone Daidzin Genistin	
Callus color		Red	Red	Red	Red	Red	Red	Blue-purple	Orange	Red	White	Yellow-green
Medium		MS	MS	MS	MS	MB5	MB5	MS	MS	MS	MS	MS
Carbon source		Sucrose	Sucrose	Sucrose	Sorbitol	Sucrose	Sucrose	Sucrose	Sucrose	Sucrose	Sucrose	Sucrose
Auxin ($mg\,l^{-1}$)		2,4-D 1.0	2,4-D 0.1	NAA 2.0	NAA 2.0	NAA 2.0	2,4-D 1.0	NAA 2.0	NAA 2.0	2,4-D 0.5	NAA 2.0	NAA 2.0
Cytokinin ($mg\,l^{-1}$)		BA 0.1	BA 0.1	BA 0.2	BA 0.2	BA 0.2	BA 0.1	BA 0.2	BA 0.2	BA 0.2	BA 0.2	BA 0.2
Times of subculturing												
Before habituation		139	25	58	209	62	163	185	139	48	39	39
During habituation												
Dilution level of auxin and cytokinin	1/2	—	—	—	—	6	—	—	—	4	4	4
	1/4	—	—	—	—	6	10	—	—	4	3	3
	1/8	—	—	—	—	12	23	—	—	4	8	4
	1/16	—	—	—	—	15	9	—	—	—	11	—
	1/32	—	—	—	—	12	10	—	—	—	3	—
	1/64	—	—	—	—	22	9	—	—	—	5	—
	1/128	—	—	—	—	4	8	—	—	—	7	—
	1/200	—	—	—	—	7	10	—	—	—	—	—
	1/400	—	—	—	—	11	21	—	—	—	—	—
	1/800	—	—	—	—	6	10	—	—	—	—	—
	1/1600	—	—	—	—	6	64	—	—	—	—	—
	1/3200	—	—	—	—	—	15	—	—	—	—	—
	1/6400	—	—	—	—	—	21	—	—	—	—	—
After habituation		246	362	337	186	248	—	159	205	308	179	209
Amount		385	387	395	395	417	373	344	344	368	259	259
Phytochemical content** for dry weight		14.5 ± 2.24 ($mg\,g^{-1}$)	18.8 ± 3.07 ($mg\,g^{-1}$)	13.9 ± 2.17 ($mg\,g^{-1}$)	3.54 ± 0.69 ($mg\,g^{-1}$)	16.3 ± 2.97 ($mg\,g^{-1}$)	0.72 ± 0.26 ($mg\,g^{-1}$)	2.74 ± 0.63 ($mg\,g^{-1}$)	574 ± 108 ($\mu g\,g^{-1}$)	9.54 ± 1.95 ($mg\,g^{-1}$)	0.83 ± 0.09 ($mg\,g^{-1}$)	2.77 ± 0.49 ($mg\,g^{-1}$)

* Japanese name.

** The values are presented as means ± SE (n=3). Antocyanin content was calculated as cyanidin 3-*O*-glucoside. Betacyanin content was calculated as amaranthine. Carotenoid content was sum of lycopene and β-carotene. Isoflavone content was sum of daidzein and genistein.

cells continued to grow and produce betacyanin on phytohormone-free medium (Table 1, Figure 1H).

For the cell line derived from the soybean hypocotyl, culture with sequential reduction of the phytohormone concentration was conducted in a similar manner to that adopted for the cell line from the *Amaranthus* sp. A cell line that required no phytohormone was eventually obtained from the white cell line by reducing the phytohormone concentration to 1/128th of the original value (0.0156 mg l^{-1} 2,4-D, 0.0156 mg l^{-1} BA) (Table 1, Figure 1I). However, a cell line requiring no phytohormone was obtained from the yellow-green cell line on reducing the phytohormone concentration to be one-eighth of the original value (0.25 mg l^{-1} 2,4-D, 0.025 mg l^{-1} BA) (Table 1, Figure 1J). The appearances and colors of these cell lines were similar to those before habituation.

All the resulting cell lines that required no phytohormones were subsequently subcultured (Table 1).

Phytochemical production by cell lines requiring no exogenous phytohormone

The anthocyanin content of the cell line derived from the cherry filament was 18.8 mg g^{-1} dry weight, which was higher than that of the corresponding cell line derived from the petal (Table 1). The major anthocyanin component of the cell line from the cherry petal was cyanidin glucoside (Figure 2A), whereas were two components were present in the cell line from the filament (Figure 2B). The content of cyanidin 3-*O*-rutinoside (Figure 2B, peak B2) in the cell line from the filament was less than that of cyanidin glucoside (Figure 2B, peak B1), unlike the case with other organs of cherry (Figure 3). The anthocyanin concentration in peach cells was four-fold higher in case of the cell line for which sucrose was used as the carbon source than in that of the cell lines for which sorbitol was used (Table 1). The major anthocyanin in both cell lines was cyanidin glucoside (Figure 2C, D).

The anthocyanin concentration in the strawberry cell line was 16.3 mg g^{-1} dry weight, which was higher than that of cherry cell line derived from the filament (Table

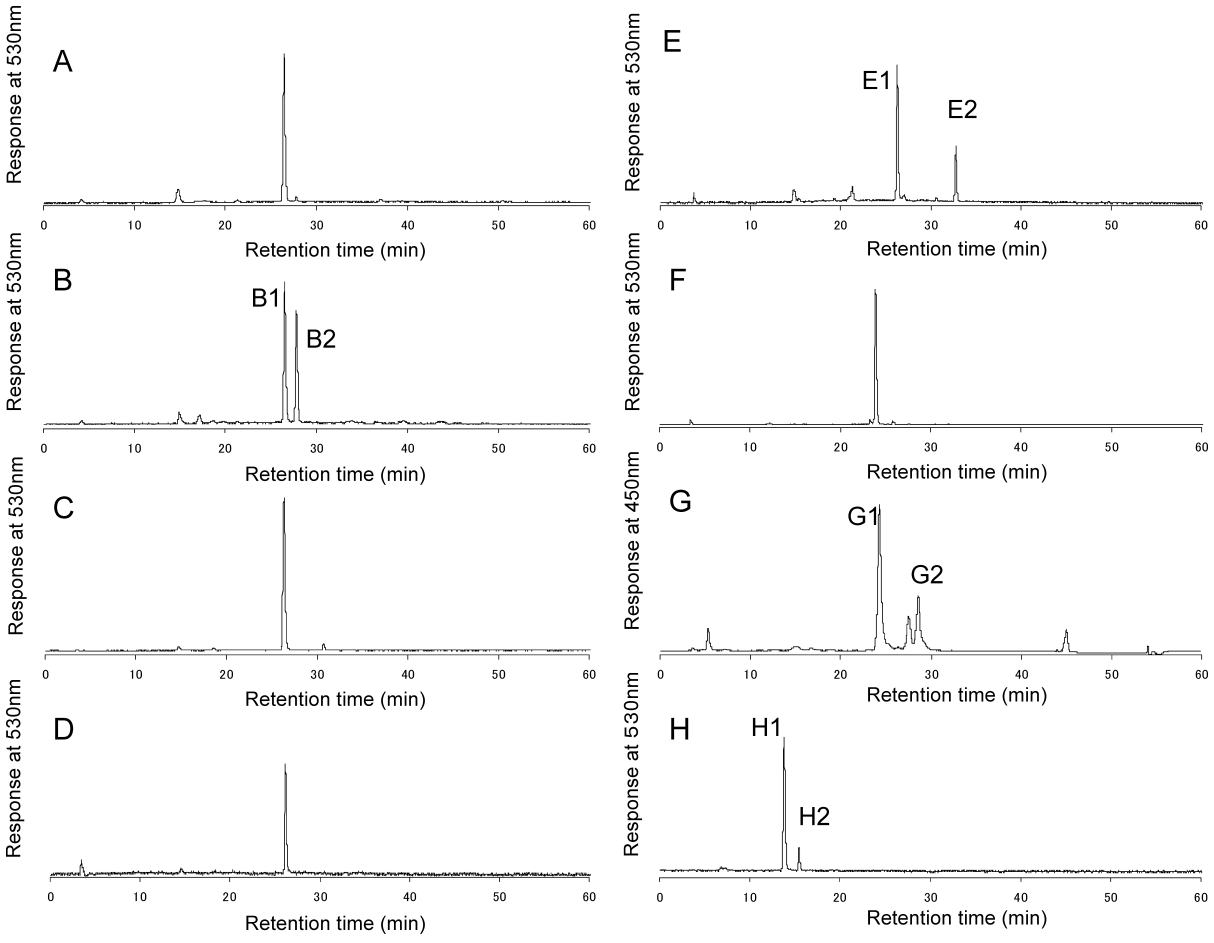

Figure 2. HPLC chromatograms of cultured cells extracts. (A) cherry (origin, petal) cells at 530 nm, (B) cherry (origin, filament) cells at 530 nm, (C) peach cells (carbon source, sucrose) at 530 nm, (D) peach cells (carbon source, sorbitol) at 530 nm, (E) strawberry cells at 530 nm, (F) pear cells at 530 nm, (G) orange Asiatic cells at 450 nm, (H) *Amaranthus* sp. cells at 530 nm.

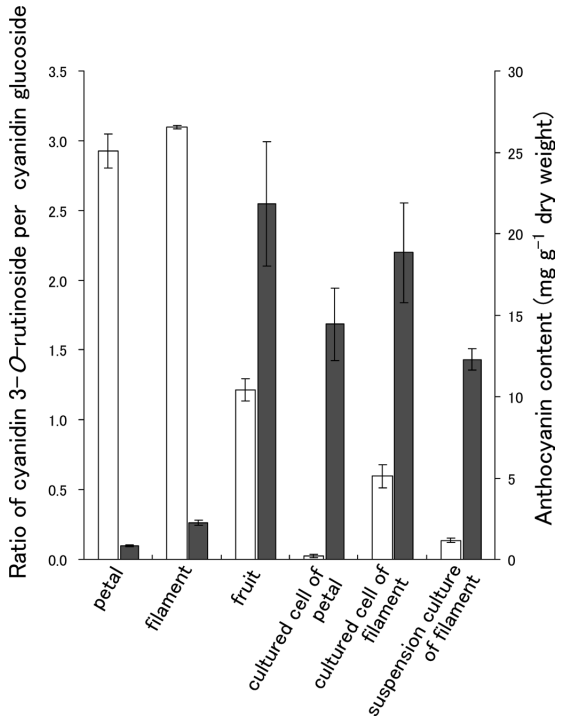

Figure 3. Ratio of cyanidin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside, and anthocyanin content in organs and cultured cells of cherry (*Prunus yedoensis*). The ratio is presented as the peak area of cyanidin 3-*O*-rutinoside and cyanidin 3-*O*-glucoside detected at 530 nm in HPLC (—□—). Anthocyanin content was calculated as cyanidin 3-*O*-glucoside (—■—). The values are presented as means ± SE (n=3).

1, Figure 1E). The major anthocyanin components were cyanidin glucoside (peak E1) and peonidin glucoside (Peak E2), in agreement with our previous report (Asano et al. 2002).

Cultured cell of pear did not become habituated to a lack of phytohormones, and their anthocyanin content was lower (0.72 mg g^{-1} dry weight) than that of cells grown before the hormone concentration of the medium was reduced (Table 1, Figure 1K, L). There was only a single anthocyanin component (Figure 2F), which was similar to the anthocyanin extracted from the anthers of pear.

The carotenoids present in the orange cultured cells of the Asiatic dayflower were identified by comparison with reference substances. Peak G1 corresponds to lycopene, and peak G2 corresponds to β-carotene. The total carotenoid content was 573 μg g^{-1} dry weight (Table 1, Figure 2G).

In the case of cultured cells from the *Amaranthus* sp., two components (corresponding to peaks H1 and H2) were found by HPLC analysis of betacyanins in the cultured cells (Figure 2H). These two components were also found in the leaves of the *Amaranthus* sp. (data not shown).

The isoflavone content of yellow-green cultured cell line of soybean was more than three-fold that in the white cells (Table 1).

In addition to the two major anthocyanin components observed in the HPLC chromatogram of extracts from cultured cells of the Asiatic dayflower, several minor components were also present (Figure 4B, F). These anthocyanin peaks differed in their retention times and peak spectra from those of the anthocyanins present in the petals of blue Asiatic dayflower (Figure 4B, F). Furthermore, no component that matched the flavonoid in the petals (Figure 4C, G) was found in the extract of the cultured cells (Figure 4D, H).

Suspension culture of cultured cells of cherry

Culture in MS liquid medium of the cherry cell line derived from the filament and requiring no exogenous phytohormone was compared with culture in MB5 medium. The cell yield was somewhat lower in MB5 medium than in MS medium, but the anthocyanin production was over 1.5-fold higher (Figure 5). When the strain was subcultured continuously in the MB5 liquid medium, the anthocyanin concentration in the cell was found to vary. It was about 37 mg g^{-1} dry weight at the beginning of culture and then decreased rapidly (Figure 6). It rose again to about 17 mg g^{-1} dry weight during the period between the 10th and 12th passage but decreased on further culture. After the 25th passage, however, the content tended to increase again gradually (Figure 6).

In addition, the continuous subculture in darkness of the tenth suspension culture of cherry cells was examined. In this case, the anthocyanin concentration did not decrease markedly after one passage, but it subsequently decreased gradually on subculturing in darkness. After the fifth passage of culture in darkness, the anthocyanin concentration approached zero (Figure 6).

The effects of the inoculation amount on the growth rate in suspension culture were examined by culture using a 250-ml Erlenmeyer flask containing 50 ml of fresh MB5 medium. Inoculation with 5 ml of cell suspension (equivalent to 10% of 50 ml) resulted in an increase to about 80% of the mass of dried cells produced by a normal-sized inoculation (10 ml) after two weeks. Additionally, growth was confirmed when 1 ml of cell suspension (2% of 50 ml) was used as the inoculant (Figure 7).

The average doubling time for cherry cells in suspension culture in a 250-ml Erlenmeyer flask was 4.16 ± 0.31 days (Figure 7).

Discussion

Some previous studies have been made on the production of anthocyanins by cultured cells of cherry; for example, cyanidin glucoside (10 μg g^{-1} fresh weight) has been produced from cultured cells of the leaf of *Prunus* ×

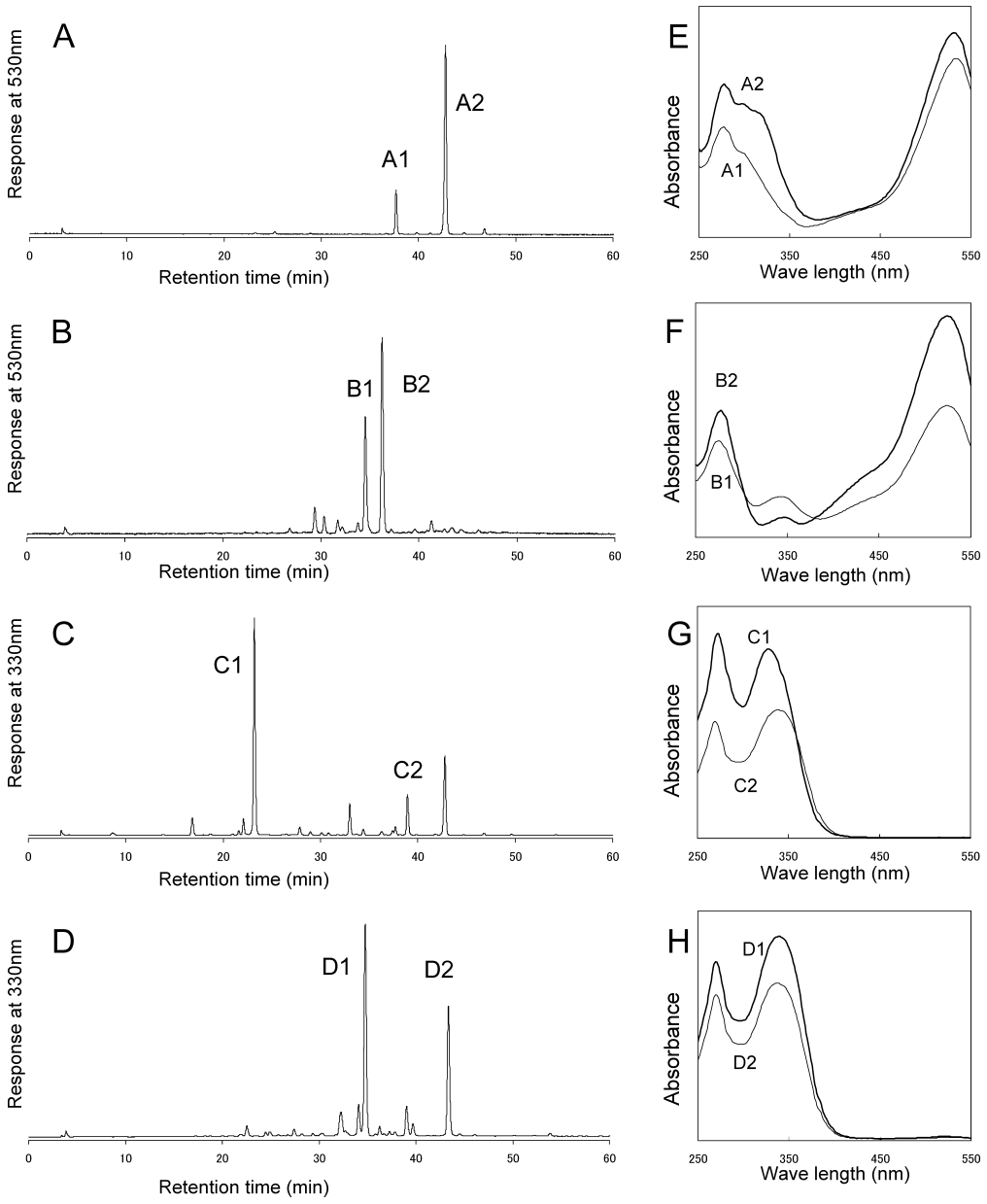

Figure 4. HPLC chromatograms and peak spectra of Asiatic dayflower petal and cultured cells. (A) chromatogram of blue petal at 530 nm, (B) chromatogram of cultured cell at 530 nm, (C) chromatogram of blue petal at 330 nm, (D) chromatogram of cultured cell at 330 nm, (E) spectrum of peaks A1, A2, (F) spectrum of peaks B1, B2, (G) spectrum of peaks C1, C2, (H) spectrum of peaks D1, D2. Absorbance of peak spectra with a photodiode array detector at optimal magnification.

yedoensis, the same species as that used in this study (Ishikura et al. 1989). Cyanidin glucoside and cyanidin 3-*O*-rutinoside (42 μg g^{-1} fresh weight) have also been produced from cultured cells of sour cherry, a related species (Blando et al. 2004).

The anthocyanin content of our cultured cherry cells was 18.8 mg g^{-1} dry weight, which is more than those previously reported, but is not as high as that in the fruit of *Prunus* × *yedoensis* (Figure 3). The anthocyanin composition of our cultured cells of cherry differed widely depending on whether the cell line was derived from the petal or from the filament. The cyanidin 3-*O*-rutinoside content of tissues of the petal and the filament is almost three times that of cyanidin glucoside, but this ratio was not reflected in the ratio for the two corresponding cultured cell lines (Figure 3). This may be the result of differentiation of cell lines during selection for anthocyanin-producing lines, thereby favoring cells that produce cyanidin 3-*O*-rutinoside over those that do not.

On the basis of HPLC analysis, the anthocyanin-producing cell line from peach fruit contained cyanidin glucoside as a major anthocyanin component, in agreement with a report on the components of the fruit

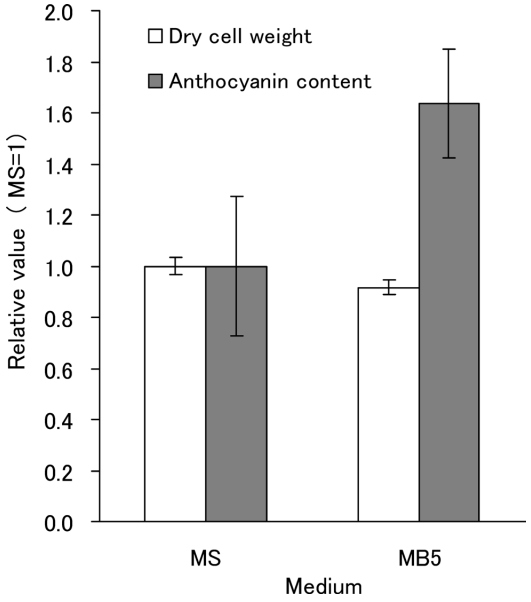

Figure 5. Effect of medium on cell growth and anthocyanin content in suspension culture of cherry filament cells. Cell suspension cultures in MB5 medium without phytohormones were derived from six times' suspension cultured in MS medium without phytohormones. Then 10 ml of old cell suspension was transferred to new 50 ml of MB5 medium without phytohormones and cultured 2 weeks. Dry cell weight and anthocyanin contents are expressed per flask. The values are presented as means ± SE (n=3).

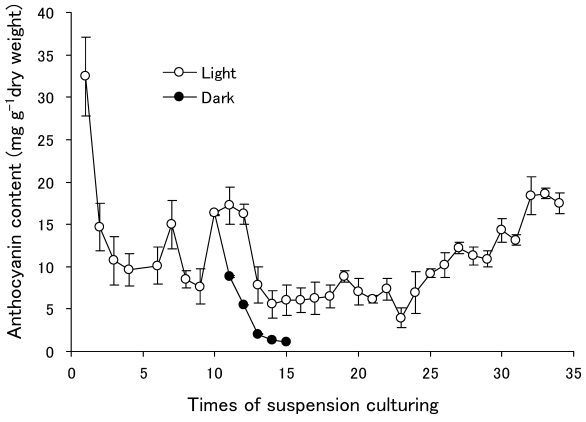

Figure 6. Change of anthocyanin content in suspension cultured cell of cherry filament. 10 ml of old cell suspension was transferred to new 50 ml of MB5 medium without phytohormones and cultured 2 weeks. Anthocyanin contents are expressed per gram of dry cell weight. The values are presented as means ± SE [n (light)=3, n (dark)=2].

(Tomas-Barberan et al. 2001). The content of the anthocyanin was 13.9 mg g^{-1} dry weight, which was more than that in peach fruit (0.41 mg g^{-1} dry weight) (Wu et al. 2006) or peel (0.34 mg g^{-1} fresh weight) (Tomas-Barberan et al. 2001). Moreover, a cell line that was incubated with sorbitol as the carbon source (Bianco et al. 2000; Fayek and Wayne 1979) was found to have a lower anthocyanin concentration than the cell line incubated on sucrose as the carbon source. This result, which has not previously been reported the literature, is

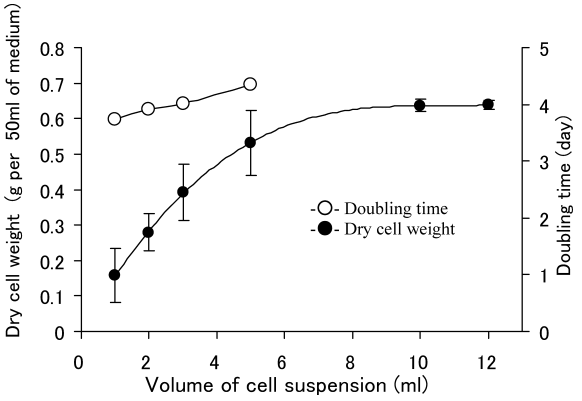

Figure 7. Effect of transferred cell suspension volume on increased cell growth, and on doubling time. Cell suspension cultures were derived from cells of cherry filaments. Each volume of old cell suspension was transferred to new 50 ml of MB5 medium without phytohormones and cultured 2 weeks. Dry cell weights are subtracted and expressed as the transferred cell weight (—●—) . The values are presented as means ± SE (n=3). The doubling time of cherry suspension culture (—○—).

of particular interest.

The anthocyanin concentration in cells from strawberry cv. Nyoho was 14.1 mg g^{-1} dry weight, which was more than that in the cell line of strawberry cv. Shikinari (10 mg g^{-1} dry weight) (Nakamura et al. 1999).

The anthocyanin component in the cultured pear cells was presumed to be cyanidin 3-*O*-galactoside (Steyn et al. 2004; Timberlake and Bridle 1971). The anthocyanin content was much lower than that before the start of habituation (Figure 1K, L).

Therefore, the contents of phytochemicals in the cells of all the plant cell lines that we examined decreased during habituation. Moreover, the components of the phytochemicals did not change markedly. Nevertheless, after habituation, growth and the production of phytochemicals recovered as a result of visual selection and subculturing (Asano et al. 2002) (Table 1, Figure 1A–H). Therefore, the effect of habituation is to decrease growth and phytochemical production.

The production of a blue pigment by plant cell culture is exemplified by the production of trichotomine from cultured cells of *Clerodendron trichotomum* Thunb. (Koda et al. 1992). Because no blue anthocyanins are currently available on the market for use as foods colorants, we targeted blue anthocyanins in our experiments. We obtained cells with a blue appearance from cultured cells of the Asiatic dayflower. However, analysis of the anthocyanins in the cells showed that they differed from those in the blue petals of Asiatic dayflower (Figure 4A, B). Malonylawobanin [delphinidin 3-(6″-(*E*)-p-coumaroylglucoside)-5-(6″-malonyl-glucoside)] (Kondo et al. 1991), which is present in the petal, could not be identified in the cultured cells. From a comparison of the spectra, the anthocyanin in the

cultured cells was presumed to lack the β-coumaric acid moiety (Figure 4E, F). In addition, the blue color of malonylawobanin requires flavocommelin (swertisin 4′-*O*-glucoside) (Shiono et al. 2008), but no flavocommelin was present in the cultured cells, where the only components with similar spectra to those of flavonoids were in the petals of the Asiatic dayflower (Figure 4G, H). Consequently, it was possible to select blue-violet cells by visual cell selection. However, because our selection method was simple and involved no component analysis, we judge that it is difficult to establish a cell line that produces both the components necessary to produce a blue color. Moreover, two cell lines, one producing an anthocyanin and the other producing carotenoids, were obtained from a single cultured cell line of Asiatic dayflower cultured cells. The total carotenoid content of the Asiatic dayflower cell line was 574 $\mu g\,g^{-1}$ dry weight, which is the same as that in carrot root, but is less than that in callus of carrot (1438 $\mu g\,g^{-1}$ dry weight) (Nishi et al. 1974). A similar behavior with cells that produce anthocyanins and carotenoids has been reported in the case of *Vaccinium ashei* Reade (Nawa et al. 1993). We therefore consider it to be highly likely that cell lines that produce different phytochemicals appear during cell selection.

The production of betacyanin by cell culture has been reported for red beet (28 $\mu mol\,g^{-1}$ dry weight) (Girod et al. 1991) and for *Portulaca* sp. (9 $mg\,g^{-1}$ dry weight) (Bhuiyan et al. 2002), and the effects of the nature of the medium on the production of betacyanin have been studied (24 $mg\,g^{-1}$ dry weight) (Akita et al. 2001, 2002). In all these cases, however, phytohormones were added to the medium. A cell line requiring no phytohormone has been established for cultured cells of sugar beet (Kevers et al. 1981, 1996), but these cells did not produce betacyanin. We succeeded in establishing a cell line that requires no phytohormone and produces betacyanin (9.5 $mg\,g^{-1}$ dry weight).

The production of isoflavones in cultured cells of soybean has been identified in a continuous suspension culture that required phytohormones (Ames and Worden 1997); in this case, daidzein was produced at 200 $\mu g\,g^{-1}$ and genistein at about 100 $\mu g\,g^{-1}$. Our yellow-green cultured cells of soybean produced 2.77 $mg\,g^{-1}$ dry weight of isoflavones, which is comparable to the isoflavone content of soybean seeds (Meksem et al. 2001, Nakamura et al. 2001). We did not monitor the isoflavone content of this cell line during selection and subculturing; we believe that it should be possible to enhance the isoflavone content of cells by monitoring their isoflavone content and selecting accordingly.

To summarize, we have succeeded in establishing habituated cell lines of various cultured cells of woody and herbaceous plants and of dicotyledonous and monocotyledonous plants. Calli were induced from petals, filaments, fruits, leaves, and hypocotyls of these plants. The resulting cell cultures produced anthocyanins, betacyanin, carotenoids, and isoflavones. We also examined the effects of using MS and modified B5 (MB5) media (which contain different ratios of ammonium and nitrate), the use of sucrose and sorbitol as carbon sources, and the actions of 2,4-D, NAA, and BA as phytohormones in the medium before habituation.

The growth and anthocyanin production of pear cells were controlled by using very low concentrations of phytohormones (0.156 $\mu g\,l^{-1}$ 2,4-D and 0.0156 $\mu g\,l^{-1}$ BA). There is no previous report in the literature of any case in which such a low concentration of phytohormones controls growth and anthocyanin production in a plant cell culture.

In previous reports, indole-3-acetic acid has been detected in cells of phytohormone-nonrequiring cell lines of *Panax ginseng* (45 $\mu g\,g^{-1}$ fresh weight), *Nicotiana tabacum* (45 $\mu g\,g^{-1}$ fresh weight) (Nishio et al. 1976) and of sugar beet (1241 $\mu g\,g^{-1}$ fresh weight) (Kevers et al. 1981), and in pale cells of soybean (100 $pmol\,g^{-1}$ fresh weight) (Wyndaele et al. 1988). Cytokinin, as a ribosyl-*trans*-zeatin equivalent, has been detected in green cells of soybean (150–270 $pmol\,g^{-1}$ fresh weight) (Wyndaele et al. 1988). The existence of these endogenous phytohormones suggests that phytohormone-nonrequiring cell lines can grow without the addition of exogenous phytohormones. From these cases, we assume that endogenous indole-3-acetic acid or cytokinin is present in our phytohormone-nonrequiring cell lines.

We found no clear tendency in the ease or difficulty in establishing phytohormone-nonrequiring cell lines for various cell types and culture conditions. A point of commonality between the cultured strawberry cells, where it took a long time to establish phytohormone-nonrequiring cell lines, and the cultured pear cells, for which no such cell line could be established, is that MB5 medium was used in both cases (Table 1). We need to perform more experiments to confirm whether this phenomenon is the result of a difference in the ratio of ammonium to nitrate as the nitrogen source in the medium, or if it is incidental.

On the basis of our results, we believe that our method for obtaining phytohormone-nonrequiring cell lines by continually decreasing the concentration of exogenous phytohormones is effective, although it cannot be applied to all cultured cell lines. As a potential alternative approach to habituation and the production of phytochemicals, it may be more effective to establish habituated cell lines initially, and then to establish phytochemical-producing lines by selecting for cells that produce the target phytochemicals.

Moreover, it will be necessary to examine methods for controlling the production of phytochemicals by

phytohormone-nonrequiring cell lines by changing the culture environment, other than the phytohormones, such as the lighting conditions or the composition of the medium (Mori 1994).

The characteristics of our phytohormone-nonrequiring cherry cells that underwent long-term subculture showed that the optimal medium for anthocyanin production in suspension culture was not the same as that for solid culture. Similarly, B5 medium has been shown to be superior to Linsmaier (LS) medium in the case of the cultured strawberry cells (Mori and Sakurai 1994).

In addition, an inoculation amount in a suspension culture of as little as 2% caused growth (Figure 7). This showed that a small amount of cell suspension is sufficient for conditioning a new medium. The average doubling time of cherry suspension culture at 25°C was about four days; this is over twice that of a *Vitis* suspension cell culture (38 hours at 30°C) (Yamakawa et al. 1983). This result provides useful data with regard to large-scale anthocyanin-producing cell cultures.

In the case of our cultured cherry cells, the anthocyanin content did not remain constant after the beginning of suspension culture, and it rose and fell (Figure 6) possibly because of a lack of a selection effect among the three flasks used for culture. We selected from among the three flasks solely by visual inspection, and we used the cell suspension with the deepest color in the next subculture. The anthocyanin concentration may have decreased when no actual effect of selection occurred and increased when an effect of selection was present.

Our results also suggest that the choice of medium should be examined and that amount of inoculation when transferring cells cultured from the solid medium to suspension culture or for producing phytochemicals by mass culture needs to be optimized. The results from such studies should apply in cases other than our cultured cherry cells. Furthermore, a reduction in the concentration of the phytochemicals could occur during suspension culture, and it may be necessary to try to maintain and enhance the concentration of phytochemicals (secondary metabolites) by selection or other methods.

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