Review Plant Tissue Culture Letters, **13**(3), 249-257(1996)

Anthocyanin Production of Cultured *Euphorbia millii* Cells*

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(Accepted September 27, 1996)

Introduction

Euphorbia species have been propagated as future sources of gasoline on a large scale[1-3]. We were interested in cell culture and production of secondary metabolites in members of the genus *Euphorbia*. Therefore, we induced calluses from *E. tirucalli*, *E. millii* and *E. lathyris*, Moreover, we isolated a cell strain of *E. millii* that produced a high level of a red pigment, and mass-produced this pigment, an anthocyanin, and applied it for dyeing textiles.

Callus Induction of *E. millii*

Callus from the sterilized leaf of *E. millii* was induced under light on Murashige and Skoog's basal medium with 0.1-10 ppm of an auxin(2, 4-D or NAA) and 0.2% of a natural extract(malt extract, yeast extract or casein hydrolysate)[4]. Malt extract promoted the induction of *Euphorbia* calluses more than casein hydrolysate or yeast extract. Two distinct *E. millii* calluses were induced. They were a yellow callus on medium containing malt extract and 1 ppm 2, 4-D, and a varied-color callus on medium containing malt extract and 0.1 ppm 2, 4-D.

Selection of Cultured *E. millii* Cells that Produced a High Level of an Anthocyanin

Usually, plant cell cultures produce only small amounts of secondary metabolites, however, cell

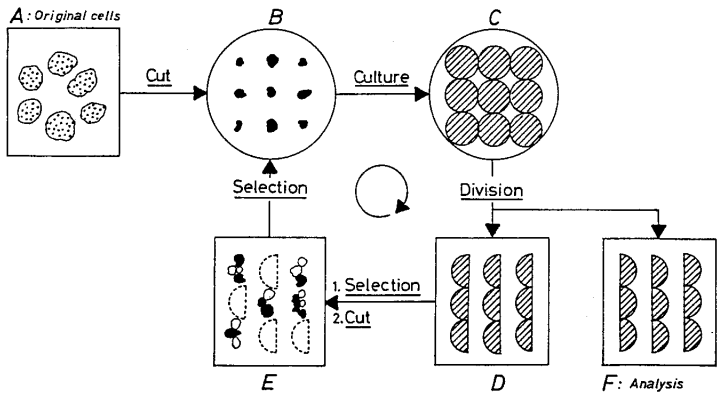

Fig. 1 Outline of the selection method.

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* **The 1996 Technology Award of the Japanese Society for Plant Cell and Molecular Biology was given to the studies described in this reviews.**

strains containing amounts of secondary metabolites greater than those found in intact plants have been isolated by clonal selection. There are two questions with respect to clonal selection: 1) How can we determine whether the high productivity of the desired metabolite in the selected cell strain is stable? 2) How long must we continue clonal selection in order to obtain stable cell strains that produce the desired metabolites? We have been interested in resolving these questions and establishing a strain that produce a high level of a secondary metabolite[5,6].

The selection method, small cell-aggregate selection, is outlined in **Fig. 1**. The original *E. millii* calluses whose color was mottled red and white were cut into 128 segments (about 3 mm in diameter). Each segment was coded and placed on agar medium in a sectioned Petri dish. The agar medium consisted of Murashige and Skoog's basal solution, 10^{-6} M 2, 4-D, 0.2% malt extract and 2% sucrose. The segments were cultured under light for 10 days. Each segment in a Petri dish was cut into two cell-aggregates, one (D_1) for subculture and the other (D_2) for quantitative analysis of the pigment. From the analysis of D_2, we selected the reddest D_1 cell-aggregate from each Petri dish. These selected cell-aggregates were cut into several segments which were then coded and transplanted onto fresh medium in Petri dish. The code of each segment was put into a computer data file together with the code of the mother cell-aggregate. The segments selected at each transplantation were cultured on the same medium under the above conditions. This selection procedure was repeated 28 times.

Pedigree analysis has been used in breeding programs of domestic animals and garden plants. The knowledge of parentage obtained by pedigree tracing can aid in assigning genotypes and indicate the degree of genetic diversity in breeding populations. Manually pedigree analysis is unwieldy, but when a computer is used, one can deal with a large breeding program that involves the crossing of many genotypes and data on numerous progeny. All codes for the 1588 cell-aggregates from the 7th to 28th generations were put into the data file. The pedigree of the cell-aggregates was graphed, and a cell line (a group of cell-aggregates derived from one ancestor) was tabled by the computer. The distribution rate (%) [(the number of cell-aggregates of a specific cell line/the number of total cell-aggregates) $\times 100$] for each line was calculated for each generation. A

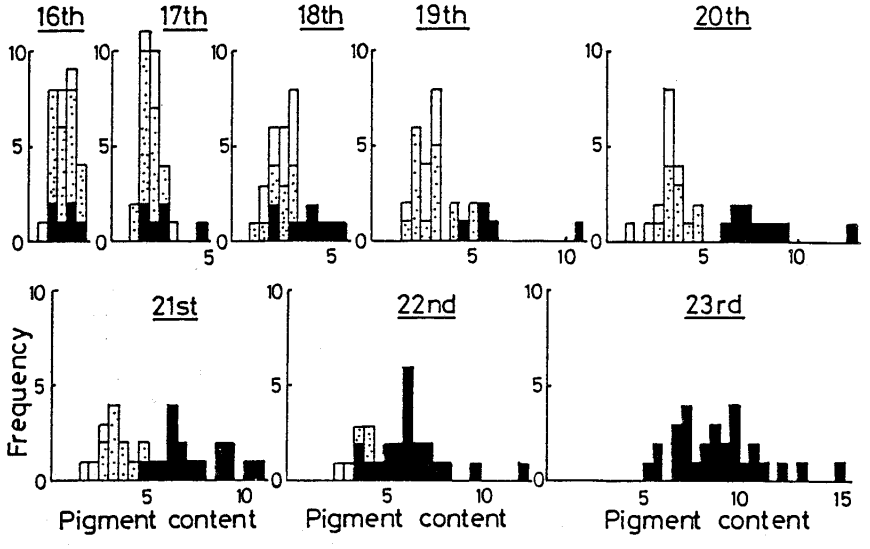

Fig. 2 Frequency distribution of cell-aggregates of the 9 A (dot area) and 9 F (black area) cell lines in histograms of the population of cell-aggregates with various pigment contents from the 16th to 23rd subcultures.

frequency distribution was then made and its mean value was calculated. This is marked in the histogram of the total cell-aggregates (**Fig. 2**). The 9 A and 9 F cell lines originated from the 9 A and 9 F cell-aggregates of the ninth subculture. These lines produced pigment-rich descendants at high frequencies. The distribution rate of the 9 F cell line was lower than that of the 9 A cell line at the 16th subculture, but equaled it at the 20th generation. This rate gradually increased and reached 100% at the 24th generation, which means that all the aggregates after the 24th generation were derived from the 9 F cell-aggregate. We also showed that the the 9 F cell line was comprised of the group of cell-aggregates that showed high pigment content in the histograms after the 18th generation.

A random sample from the D_2 population was prepared for quantitative analysis of the red pigment. The absorbance of the acidic MeOH solution of each segment was measured at 530 nm; then the pigment content [absorbance at 530 nm/fresh weight (mg) of the cell-aggregate] was calculated. A frequency distribution of cell-aggregates with various pigment contents was made. Two characteristic terms, the mean (C) and maximum (Cmax) values, were calculated. The reddest cell-aggregates were selected continuously from each of the 28 subculture generations of the original mottled red *E. millii* callus. The mean value (C) for the pigment content increased nearly threefold from the 16th to 22nd generations. It was 7.46 ± 0.56 after the 23rd generation, which was seven times higher than the pigment content (1.05) of the original callus. The maximum value (Cmax) increased nearly threefold from the 16th to 19th generations, then was 12.96 ± 2.36 after the 20th generation. We found that the mean value for the anthocyanin content in cell-aggregates of *E. millii* became stable after 24 clonal selections. If the mean value for the content of secondary metabolites is stable in a population of cell-aggregates chosen by successive clonal selection, and if all the cell-aggregates are derived from one cell-aggregate, they should consist of cells with high and stable productivity of secondary metabolites. These results show that we succeeded in isolating and culturing a cell strain with a high and stable pigment content from *E. millii* callus after 24 successive clonal selections.

We believe that during early generations cultured cells are heterogeneous in their production of pigments and that they contain variant cells producing high levels of pigment. Now, for 13 years after selection, the strain maintains the capability of pigment production with a high level that is equal to the first.

Suspension Cultures of Variant *E. millii* Cells

There have been many reports on anthocyanin production in cultured plant cells. No reports have appeared on the industrial application of anthocyanins obtained from cultured cells. We investigated to mass-produce an anthocyanin by suspension cultures of *E. millii* cells[7,8].

E. millii cells that produced a high level of an anthocyanin cultured for 10 days in liquid media containing inorganic solutions of Linsmaier-Skoog (LS), Murashige-Skoog (MS), Gamborg (GA), Nitsch-Nitsch (NN), Heller (HE), or White (WH). The anthocyanin production in GA medium was higher than in other media. Accordingly, GA solution was used as the basal inorganic solution in the following experiments. The optimum concentration for cell growth was 5% ; on the other hand, that for anthocyanin content was 7%. Nine percent sucrose diminished cell growth. The cells died in the absence of sucrose.

A combination of BA and either 2, 4-D or NAA influenced the anthocyanin production. The cell growth at the higher concentration (10^{-5} M) and the anthocyanin content at the lower concentrations (10^{-6} and 10^{-7} M) of 2, 4-D increased. The optimal combination of 2, 4-D and BA was 10^{-6} and 10^{-8}

M, respectively. The cell growth increased at the middle concentration (10^{-5} M) and the anthocyanin content at the lower concentrations (10^{-5} and 10^{-6} M) of NAA. The optimal combination of NAA and BA was 10^{-6} M and 10^{-9} M, respectively. The BA concentration (10^{-10} to 10^{-7} M) slightly influenced the anthocyanin production irrespective of the kind of auxin. More than 10^{-6} M BA greatly decreased both the cell growth and anthocyanin production.

Anthocyanin production was better promoted by 30 mM total nitrogen than by 15 and 60 mM. This level is equal to that in the standard GA medium. The optimal ratio of NH_4^+ to NO_3^- for anthocyanin production was 1/16. Anthocyanin production and cell growth were inhibited by high concentrations of NH_4^+. The main ion components besides the nitrogen sources were Ca^{2+}, Mg^{2+}, Fe^{2+}, and Cu^{2+} as cations, and PO_4^{3-} and SO_4^{2-} as anions. The higher concentrations of Fe^{2+} and SO_4^{2-} reduced production. Mg^{2+}, Cu^{2+}, and PO_4^{3-} at the concentrations used did not influence its production.

We established EM medium (**Table 1**) for optimum anthocyanin production according to the above results. The anthocyanin content of cells cultured in EM medium on the rotatory shaker was also higher: the anthocyanin production in EM medium (32 mg$\cdot l^{-1}\cdot$day^{-1}) was 4. 5 times higher than that in MS medium.

E. millii cells which had been cultured in the EM medium on the rotatory shaker were transferred into fresh EM medium in an enveloping-shaped film culture vessel[8]. Production of anthocyanin increased with decreased thickness. The optimum temperature for anthocyanin production under the illumination was at 22°C. The anthocyanin production (*ca.* 60 mg$\cdot l^{-1}\cdot$day^{-1}) in film vessel with 25 μm thickness was increased with mild agitation by a rotatory shaker under 3500 lux fluorescent

Table 1. Composition of MS, GA, and EM media.

	MS Medium	GA Medium	EM Medium
KNO_3	1, 900 mg/L	3, 000 mg/L	2, 648 mg/L
NH_4NO_3	1, 650	0	141
$MgSO_4$ 7 H_2O	370	500	500
$CaCl_2$ 2 H_2O	440	150	150
KH_2PO_4	170	0	0
KCl	0	0	264
NaH_2PO_4 2 H_2O	0	150	150
$FeSO_4$ 7 H_2O	27. 8	27. 8	13. 9
Na_2EDTA	37. 3	18. 7	18. 7
$MnSO_4$ 4 H_2O	22. 3	10	10
$ZnSO_4$ 7 H_2O	8. 6	2	2
$CuSO_4$ 5 H_2O	0. 025	0. 025	0. 025
$CoCl_2$ 6 H_2O	0. 025	0. 025	0. 025
KI	0. 83	0. 75	0. 75
H_3BO_3	6. 2	3	3
Na_2MoO_4 2 H_2O	0. 25	0. 25	0. 25
Inositol	100	100	100
Nicotinic acid	0. 5	1. 0	0. 5
Pyridoxine HCl	0. 5	1. 0	0. 5
Thiamine HCl	1	10	1
Glycine	2	0	2
Sucrose	20, 000	20, 000	50, 000
Malt extract	2, 000	2, 000	0
2, 4-D	10^{-6}M	10^{-6}M	10^{-6}M
BA	0	0	10^{-8}M

light.

E. millii cells which had been cultured in the EM medium on the rotatory shaker were transferred into fresh EM medium in a 30 *l* jar-fermentor with inner fluorescent lamps. They were maintained at 0.5, 1.0 or 1.5 *l*/min. of air flow rate at 26°C under light for 10 days. Dissolved oxygen concentration (DO) at 1.0 *l*/min. of flow rate was decreased as culturing and best production (*ca.* 50 mg·l^{-1}·day^{-1}) among them was obtained. In controlling DO at about 8 ppm by the air flow, the anthocyanin production reached the maximum (*ca.* 90 mg·l^{-1}·day^{-1}).

Besides, we selected the strain that produced anthocyanin in the dark with equal level of that under light.

Isolation and Identification of Pigments from Cultured Cells and Intact Plant of *E. millii*

Phytosterols and fatty acids were isolated from the hexane extract of cultured *E. millii* cells and were identified as sitosterol, stigmasterol, campesterol, cholesterol, palmitic acid and linoleic acid by GC-MS analyses[4,9].

The acidic extract from dried *E. millii* cells contained two pigments. Treatment of organic solvent separated to red and pale yellow pigments. A red pigment isolated from aqueous layer was subjected twice to preparative HPLC to give an anthocyanin (EM-1). A pale yellowish pigment isolated from organic layer was subjected twice to preparative TLC to give a flavonol (EM-2).

EM-1 was hydrolyzed by 20% HCl in MeOH solution to obtain its aglycon and sugar. The aglycon and authentic anthocyanidins were submitted to TLCs. The Rf values of the aglycon were consistent with those of cyanidin. The sugar and authentics were submitted to TLC and HPLC. The sugar was identified as arabinose by comparison with the Rf and Rt values of the authentic sugars. EM-1 had a molecular ion at m/z 419 and a fragmentation ion at m/z 287, where there was a peak of the molecular ion of cyanidin, formed by elimination of a pentose from EM-1. The NMR spectrum of EM-1 suggested that EM-1 was cyanidin arabinoside. The chemical shifts of signals of EM-1 between 6.6 and 8.8 ppm were identical with these of cyanidin. The anomer proton of the sugar moiety had a chemical shift of 5.3 ppm (*d*, $J=6.3$ Hz). The sugar protons had chemical shifts of 3.4-4.2 ppm. These shifts proved that the sugar moiety was arabinose. The UV spectrum of Em-1 had two λ max at 531 and 282 nm, $E_{440}/E_{max}=21\%$, and $E_{uvmax}/E_{max}=56\%$. Cyanidin-3-arabinoside had two λ max at 530 and 285 nm, $E_{440}/E_{max}=24\%$, and $E_{uvmax}/E_{max}=68\%$; on the other hand, cyanidin-7-arabinoside had two λ max at 533 and 275 nm, $E_{440}/E_{max}=27\%$, and $E_{uvmax}/E_{max}=131\%$[10]. These results showed that EM-1 was cyanidin-3-arabinoside shown in **Fig. 3**.

Cultured plant cells produce many anthocyanins: cyanidin-3-glucoside (*Crinum defixum*[11], *Haplopappus gracilis*[12], *Helianthus tuberrosus*[13], *Populus* sp.[14] and *Vitis* sp.[15]), Cyanidin-3-(6″-malonylglucoside) (*Centaurea cyanus*[16]), pelalgonidin-3-glucoside (*Crinum defixum*[11]), peonidin-3-glucoside (*Vitis* sp.[15]), cyanidin-3,5-diglucoside (*Haplopappus gracilis*[12], *Helianthus tuberrosus*[13],

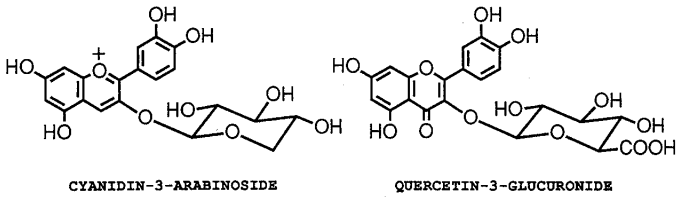

Fig. 3 Isolated flavonoids, cyanidin-3-arabinoside and quercetin-3-glucuronide from cultured *Euphorbia millii* cells.

Malus pumila[13] and *Rosa multiflora*[13]), delphinidin-3, 5-diglucoside (*Rosa multiflora*[13]), malvidin-3, 5-diglucoside (*Daucus carota*[13]), peonidin-3, 5-diglucoside (*Vitis* sp.[15]), malvidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside (*Petunia hybrida*[17]) and petunidin-3-(*p*-coumaroyl)-rutinoside-5-glucoside (*Petunia hybrida*[17]). All anthocyanins from these cultured cells are glucosides. This is the first report of the isolation of an anthocyanidin arabinoside from cultured plant cells.

Color change of EM-2 solution by NaOH and its UV spectral data indicated that EM-2 is a flavonol. EM-2 was hydrolyzed by glucuronidase to obtain its aglycon. The aglycon and authentic flavonols were submitted to TLCs. The Rf values of the aglycon were consistent with those of quercetin. EM-2 had a molecular $+H^+$ ion at m/z 479 and a fragmentation ion at m/z 303, where there was a peak of quercetin $+H^+$ ion, formed by elimination of glucuronic acid from EM-2. EM-2 was proved to be composed of quercetin and glucuronic acid. The NMR spectrum of EM-2 suggested that EM-2 was quercetin-3-glucuronide. The chemical shifts of signals of EM-2 between 6.2 and 7.8 ppm were identical with those of quercetin. The anomer proton of sugar moiety had a chemical shift of 5.46 ppm (d, $J=7.7$ Hz). The other sugar protons had chemical shifts of 3.5-3.9 ppm. The carboxylic proton had a chemical shift of 12.20 ppm. These shifts proved that the sugar moiety was glucuronic acid. These results showed that EM-2 was quercetin-3-glucuronide shown in **Fig. 3**.

Cultured plant cells produce many flavonols: baicalein-7-glucuronide (*Scutellaria baicalensis*[18]), isorhamnetin (*Cicer arietinum*[19]), kampferol (*Cicer arietinum*[19]), quercetin (*Cicer arietinum*[19]) and *Crotalalaria junicea*[20]), quercetin-3-glucuronide (*Anethum graveolens*[21]), quercetin-3-glucoside (*Parthenocissius tricusbidata*[22]), rutin (*Stevia rebaudiana*[23]) and wogonin-7-glucoside (*Scutellaria baicalensis*[18]). These flavonols from cultured plant cells are mainly glucosides. We first report the isolation of a flavonol glucuronide from cultured cells.

Cultured *E. millii* cells produced high levels of two unique flavonoids, cyanidin-3-arabinoside and quercetin-3-glucuronide in plant cell cultures. These have similar chemical structure and biosynthetic pathway. This means that the selection of red cells leads to an increase not only the anthocyanin pathway but also the flavonol pathway.

The acidic MeOH extract from intact flowers of *E. millii* was submitted with EM-1 and EM-2 to TLC and HPLC. The chromatographic results showed that the flowers produced one anthocyanin and one flavonol, and the Rf and Rt values were identical with those of EM-1 and EM-2.

There were some reports on the isolation of cyanidin-3-arabinoside from intact plants: fruits of apple[10], cacao[24], *Vaccinium angustifolium*[25] and *V. macrocarpom*[26], and flowers of *Rhododendron* genus[27]. However, this is the first isolation of cyanidin-3-arabinoside from genus *Euphorbia*. While there were some reports on the isolation of quercetin-3-glucuronide from intact plants: leaves of *Euphorbia lathyris*[28], *E. retusa*[29], *E. sanctae-catharinae*[29], *Phaseolus vulgaris*[30], *Populus grasidentata*[31], *Vaccinium uliginosum*[32] and *Vitis vinifera* var. *tinctoria*[33], fruits of *Rubus idaeus*[34], and flowers of *Tamarix nilotica*[35]. Both of cyanidin-3-arabinoside and quercetin-3-glucuronide were minor components in these plants and were not common in nature.

Dyeing Silk with an Anthocyanin from Cultured *E. millii* Cells

Since ancient times mankind has used natural pigments as dyes. They are classified into three groups by source: animal, plant and mineral. Dyeing with natural plant pigments is called 'kusakizome' in Japanese. Such pigments are mainly flavonoids which fall into two groups, flavones and anthocyanins. Anthocyanins are water soluble and are contained in cell vacuoles in leaves, roots, stems, flower petals, and fruit peels. They occur in a wide range of colors, from reddish orange,

pink, red, and purple to blue. Although many kinds of anthocyanins have been isolated from natural sources, they have not been supplied industrially as textile dyes. Because kimonos dyed by the kusakizome method are very expensive in Japan, we investigated the use of this anthocyanin in the dyeing of silk[36].

Anthocyanin-producing *E. millii* cells were cultured in EM liquid medium at 26°C on a rotatory shaker under continuous light. Harvested dry cells were treated in pH 2, pH 4 or pH 6 H_2SO_4; then each of red anthocyanin solutions was filtered. Cloths of silk and wool were dyed at 80°C in the anthocyanin solution. The structures of anthocyanins are known to change with pH. The solution of cyanidin-3-arabinoside was brilliant red below pH 2. The red anthocyanin as a pyrylium cation in acid state is stable. Therefore, the cloths of silk and wool dyed with this pH 2 solution were pinkish red.

The silk material dyed with an anthocyanin was soaked in a mordanting solution of various metal salts. We used 32 kinds of metals; they were Mg, Ca, Sr, Ba, Y, Ce, Yb, Ti, V, Cr, Mo, W, Mn, Fe, Co, Ni, Ru, Rh, Pd, Ir, Pt, Cu, Ag, Au, Zn, B, Al, In, Sn, Sb, Bi and Te. Three Munsells's characters, hue, value, and chrom of silk cloth mordanted with the above metal salts were measured. The various mordants yielded a wide range of color hues. Yellowish green cloth was obtained from V, Cr, W, Ni, Sn or Bi mordanting. The cloth color was yellow from mordanting with CaO. Purple and reddish purple cloth was obtained by mordanting with Mn, Co or Mg. Mordanting of Au, Pd, Fe or Ru gave brown cloth.

Three mordanting methods were tested: prior, concurrent and post mordanting (hereafter pre-, syn- and post-mordanting, respectively). For pre- and post-mordanting, the material was soaked in a mordanting solution. The duration and temperature of syn-mordanting were those given above for dyeing. The mordanting method affected cloth color as great as mordants. Combination of mordanting methods and mordants in dyeing with this anthocyanin yielded silk cloth of many pastel colors with high value and low chrom. Pastel silks are very attractive to ladies nowadays; therefore, these were excellent results.

Dyed silk cloth was steamed in a steamer to change and stabilize its color. We found the optimum condition for steaming when we used this anthocyanin for dyeing silk.

Silk cloth was treated in a solution of Sandspace S Liquid (Sandz Co., Ltd.) to give sulfonated silk cloth. Sulfonated silk was dyed with this anthocyanin and various mordants. Sulfonation of silk cloth yielded higher chrom and greater color change from the original pink than untreatmented cloth.

Color fastness to light was assayed by JIS-L-0842. Silk cloth dyed with the solution of this red anthocyanin had better or same level of fastness to light than other natural dyes from intact plants (*Caesalpinia sappan, Gardenia jasminoides, Sophora japonica, Curcuma longa* or *Lithospermum erythrorhizon*).

Silk handkerchiefs, scarves, tablecloths and tapestries were dyed with this anthocyanin and various mordants. A formal kimono, a 'yuzenzome irotomesode', and an obi were also dyed with this anthocyanin and various mordants. This is the first report on dyeing a kimono with a pigment obtained from cultured cells. These produced pastel colors suitable for middle age ladies, therefore it will be accepted in a market with a high price.

Conclusions

(1) We induced callus from *E. millii* leaves. A strain of cultured *E. millii* cells producing high levels of a red pigment was established by a cell-aggregate selection. The level of anthocyanin

production obtained after 24 selections was 26 times that of the original cells and can be maintained over 10 years.

(2) This strain of *E. millii* was cultured in liquid medium on a rotatory shaker and in a jar fermentor in order to increase the yield of the anthocyanin. This gives the maximum production (*ca.* 90 mg·l^{-1}·day^{-1}) of anthocyanin under optimum conditions.

(3) Two flavonoids, cyanidin-3-arabinoside and quercetin-3-glucuronide, that were minor metabolites in nature, were isolated and identified from cultured *E. millii* cells.

(4) These pigments dyed silk, wool and cotton with various mordants to pastel colors. We succeeded in using them for commercialized application.

Acknowledgments

We thank Prof. Yasuyuki Yamada of Nara Institute of Science and Technology and Prof. Akira Katayama of Tokyo Kasei University for their advice on cell culture and dyeing textiles, and also thank staffs of Nippon Paint Co., Ltd. for their help.

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