Selection of a High and Stable Pigment-producing Strain in Cultured *Euphorbia millii* Cells

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Summary. A high pigment-producing strain of cultured *Euphorbia millii* cells was isolated by clonal selection. The pigment obtained was red and consisted mainly of anthocyanin. The amount of this pigment obtained after 24 selections was seven times that found in the original cells. Statistical and cell-pedigree analyses proved that this cell strain has stable productivity for this red pigment.

Key words: Clonal selection - *Euphorbia millii* - Anthocyanin - Cell-pedigree - Cultured cells

**Introduction**

Usually plant cell cultures produce only small amounts of secondary metabolites. Recently, however, cell strains containing amounts of secondary metabolites greater than those found in intact plants have been isolated by clonal selection. These are high vitamin B₆-producing strains (Yamada and Watanabe 1980), high alkaloid-producing strains (Zenk et al. 1977; Ogino et al. 1978) and high ubiquinone 10-producing strains (Matsumoto et al. 1980).

There are two questions in respect to clonal selection: 1) How can we determine whether the high productivity for secondary metabolites in selected cell strains is stable? 2) How long must we continue clonal selection in order to obtain stable cell strains that produce secondary metabolites?

We continuously selected cell strains that showed high contents of a red pigment, an anthocyanin from cultured *Euphorbia millii* cells (Yamamoto et al. 1981), and studied the stability of their productivity for this pigment with statistical and cell-pedigree analyses.

**Materials and Methods**

**Selection and Culture**

The outline of the selection method, cell-aggregate cloning, is shown in Fig. 1. Original *Euphorbia millii* calluses which produced a red pigment, cyanidin monoglucoside, were induced from leaves. The color of the induced calluses was mottled red and white. These calluses were cut into 128 segments (cell mass, ca. 3 mm) with a scalpel. Each segment was coded and placed on agar-medium (25 ml) in a sectioned Petri dish 9 cm in diameter. The agar-medium consisted of Murashige and Skoog's basal solution (Murashige and Skoog 1962), 10⁻⁶ M 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2% (w/v) malt extract, 2% (w/v) sucrose and 0.8% (w/v) agar. The segments were cultured at 28°C under fluorescent light of 6000 lux for...
10 days. Each of the 9 segments on a Petri dish was cut into two cell-aggregates; one (D1) for subculture and the other (D2) for quantitative analysis of the pigment. From the analysis of D2, we selected the reddest D1 cell-aggregate from each Petri dish. These selected cell-aggregates were cut into several segments (cell mass, ca. 3 mm). All these segments were coded and transplanted onto fresh medium in a 9-section Petri dish. The code of each segment was put into the data file of a computer together with the code of the mother cell-aggregate. Segments selected at each transplantation were cultured on the same medium under the above conditions. This selection procedure was repeated 28 times.

Statistical Analysis (Determination of Pigment Contents: the Mean and Maximum Values)

A random sample of 30 from the D2 population was prepared for quantitative analysis of the red pigment. Each segment of the sample was soaked in MeOH (4 ml) containing 2% HCl for 1 hr. The absorbance of the methanolic solution was measured at 530 nm, which was the \( \lambda_{max} \) of this pigment, 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 (class-width was fixed at 0.5). Two characteristic terms; the mean (C) and maximum (C\( \text{max} \)) values were calculated.

Cell-pedigree Analysis (Determinations of the Distribution Rate, the Frequency Distribution and the Mean Value of the Red Pigment in the Specific Cell Line)

All codes for the 1588 cell-aggregates from the 8th to 29th passages were put into the data file. The pedigree of the cell-aggregate was graphed, and a cell line (a group of cell-aggregates derived from one ancestor) was tabulated 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 frequency distribution then was made, and its mean value was calculated. This is marked in the histogram of the total cell-aggregates.

Results

The reddest cell-aggregates were selected continuously from each of the 28 subcultures of the original mottled-red, \textit{Euphorbia millii} callus. Two characteristic terms (C and C\( \text{max} \)) in the frequency distribution of cell-aggregates from the 16th to 28th passages were plotted against the pigment content in Fig. 2.

The mean value (C) for the pigment content increased nearly threefold from the 16th to 22nd passages. It came within the limit of 7.46 ± 0.56 after the 23rd passage, which was seven times higher than the pigment content (1.05) of the original callus. The maximum value (C\( \text{max} \)) increased nearly threefold from the 16th to 19th passages, then was within the limit of 12.96 ± 2.36 after the 20th passage.

The 9A and 9F cell lines originated from the 9A and 9F cell-aggregates of the 9th subculture. In this subculture, the 9A and 9F lines produced pigment-rich descendants at high frequencies. The distribution rates of these two cell lines in the population of cell-aggregates from the 16th to 24th passages is shown in Fig. 3.

The distribution rate of the 9F cell line was lower than that of the 9A cell line at the 16th subculture, but equalled it at the 20th subculture. This rate gradually increased and reached 100% at the 24th subculture. The trends of two mean values (C\( _A \) and C\( _F \)) for the pigment contents in cell-aggregates of the 9A and 9F cell lines...