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Selection of a reserpine-producing cell strain using UV-light and optimization of reserpine production in the selected cell strain

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Abstract. Cultured *Rauwolfia serpentina* calluses consisted of cell colonies that had different fluorescences under 365 nm UV-light. Fluorescence was divisible into two main color categories, yellow-green and blue-white. Two cell strains of different fluorescence were selected from calluses cultured in the dark, by the fluorescence assay. Even after the selected strains had been transferred to liquid medium, they maintained similar fluorescence. HPLC analysis showed that the yellow-green fluorescent strains produced much reserpine, whereas the blue-white strains produced much 3,4,5-trimethoxy benzoic acid. A combination of 10 μM NAA and 10 μM BA enhanced production of reserpine in the yellow-green fluorescent cell strains.

Introduction

Cultured plant cells are heterogeneous with regard to gene expression, therefore it is possible to select cell strains that produce useful metabolites. There are now many reports on the selection of cultured cell strains that give high yields of such useful compounds as berberine, nicotine, ubiquinone [1], anthocyanin, biotin and indole alkaloids [2, 3].

Selected cell strains have been obtained by various selection methods. Yamada and Watanabe [4] reported high biotin-producing cell lines of cultured *Lavendula vera* that they obtained by cell cloning and a bioassay with high concentrations of pimelic acid. Ogino et al. [5] reported the selection of high nicotine-producing cell lines of tobacco callus by single cell cloning using the cell squash method. Mizukami et al. [6] and Yamamoto et al. [7] succeeded in selecting cell lines that produce large amounts of shikonin and anthocyanin pigments using a visual selection method. High berberine-producing cell lines also have been established by cloning small cell aggregates; tyrosine, a precursor of berberine, being the selective agent used [8]. Recently, high yields of serpentine-producing cell lines have been reported, selecting cultured *Catharanthus roseus* cells by a fluorescence assay, but these strains were unstable without clonal selection [9].

Previous papers from our laboratory [10] have reported the primary selection of reserpine-producing cell strains from cultured *Rauwolfia ser-*

pentina cells under stress and the production of reserpine by metabolic regulation. The reserpine contents (0.03–0.06% dry wt) of these suspension cultures, however, were too low to use for industrial application. We had to select high reserpine-producing cell strains from the primary selected strains for the improvement of reserpine production. The study reported here deals with the secondary selection of reserpine-producing cell strains by small cell aggregate cloning using the fluorescence assay and the optimum culture conditions for reserpine production in yellow-green fluorescent cell strains.

Materials and methods

Cultured cells

Callus cultures derived from the stem of *Rauwolfia serpentina* have been maintained for more than 13 years in the dark at 25 °C on modified Linsmaier-Skoog (LS) [11] medium supplemented with 1 μM 2,4-D and 1 μM kinetin and containing KNO_3 instead of NH_4NO_3 as the nitrogen source. Although the initial cultures produced only traces of reserpine (0–0.003% of dry cell wt), we could select reserpine-producing strains from them under conditions of stress induced by altering the composition of the medium, including the hormones present. On the basis of preliminary experiments, LS medium supplemented with 10 μM NAA and 1 μM BA was used to create stress. Thereafter, the strains selected were cultured in the original growth medium. These reserpine-producing calluses were used as the source material for secondary selection with UV-light.

Selection methods

Nine pieces of small cell aggregates (2–3 mm in dia) were plated in a petri dish (9 cm in dia) containing 12 ml of agar-modified LS medium supplemented with 1 μM 2,4-D and 1 μM kinetin. After incubation in the dark for 3–4 weeks, the fluorescence of large growing cell aggregates (ca. 10 mm in dia) was checked under 365 nm UV-light. Two different colored fluorescent colonies were selected, and incubated at 25 °C. This selection cycle was repeated more than 6 times.

Qualitative analysis of the selected cell aggregates

To affirm that fluorescence can be used as an indicator for selection, we analyzed the alkaloidal components of our small cell aggregates by TLC. Fifty milligrams of freeze dried cells were shaken with 5 ml of MeOH for 3 h at 50 °C, after which the cells were centrifuged at 3000 rpm for 10 min. Treatment with MeOH was repeated once more, then the combined MeOH extracts were dried completely under a vacuum at 50 °C. The dried residue was dissolved in 3 ml of $CHCl_3$ and shaken vigorously for 15 sec.

The $CHCl_3$ extracts were separated by centrifuging them at 3000 rpm for 3 min at 4 °C, the supernatant obtained being analyzed immediately by TLC. The relative content of reserpine in the selected cells was estimated by the fluorescence density of the reserpine spot separated by TLC at 365 nm. The TLC silica layers (Kieselgel 60F-254, MERCK) were developed with MeOH-$CHCl_3$ (5:95).

Quantitative analysis of suspension cells derived from selected cell aggregates

Several promising cell aggregates obtained by secondary selection with UV-light were transferred to liquid medium. Samples for HPLC analysis were prepared by the procedure described above. The $CHCl_3$ extracts were dried thoroughly under a vacuum, and their dried residue dissolved in the solvent used for HPLC; n-hexane:ethanol:acetic acid:triethyl amine (50:50:0.3:0.4). This solution was centrifuged at 3000 rpm for 10 min at 4 °C, and its supernatant used for HPLC. Normal phase partition chromatography on a Waters Associates, model 441 liquid chromatograph was used for the HPLC analysis. The $CHCl_3$ extracts were separated through SILICA-150 (Toyo Soda Co., Ltd) in a 4.6 × 250 mm stainless steel column at a flow rate of 1.2 ml/min. The effluent was monitored at 280 nm. Reserpine and trimethoxy benzoic acid, a precursor of reserpine, were identified by comparison with the retention times of authentic standards.

Culture conditions for cell strains obtained by secondary selection

The yellow-green and blue-white cell strains selected by fluorescence have been maintained in modified LS liquid medium supplemented with 1 μM 2,4-D and 1 μM kinetin or 10 μM NAA and 10 μM BA at 25 °C in the dark, and have been subcultured at intervals of 3 weeks. The amount inoculated in 25 ml of liquid medium in a 100-ml flask was approximately 1.0 g fr. wt and in 75 ml of medium in a 300-ml flask about 2.5 g fr. wt.

Results and discussion

Selection of reserpine-producing cell strains using UV-light

The callus cultures obtained by primary selection were all the same color, but were comprised of colonies that showed different fluorescence under 365 nm UV-light. Fluorescence was divisible visually as yellow-green and blue-white. After these two kinds of fluorescent colonies had been separated from the heterogeneous callus cultures, we analyzed the alkaloids present in each type by TLC.

The fluorescence of the reserpine spot that appeared on the TLC plates under 365 nm UV-light was very intense in the yellow-green fluorescent colonies, but relatively faint in blue-white fluorescent colonies. Therefore, we investigated the yellow-green fluorescence as an indicator of reserpine

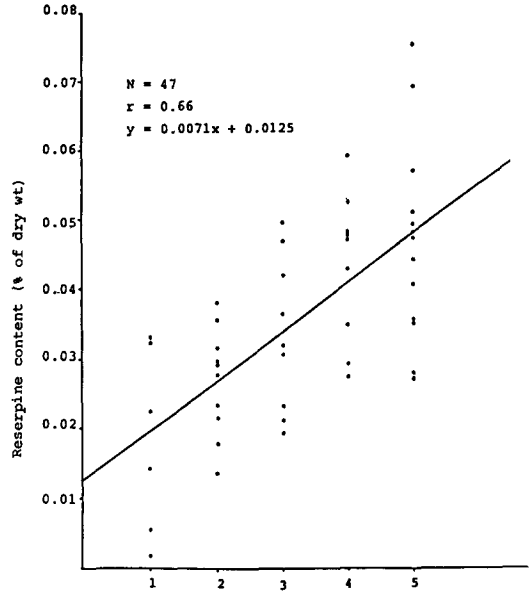

Figure 1. Correlation between reserpine content and fluorescence in cell strains selected under 365 nm UV-light. Fluorescence was classified as 1. pale violet, 2. blue-white, 3. blue yellow-green, 4. faint yellow-green and 5. yellow-green.

production for use in selecting reserpine-producing cell strains. As starting materials for secondary selection, the callus cultures obtained primary selection, which were heterogeneous in their production of secondary metabolites, were plated randomly on agar medium in a petri dish. After being incubated for 3–4 weeks, the colonies formed showed yellow-green, blue-white and pale violet fluorescence in large cell aggregates. Each fluorescent colony selected from the cell aggregates was broken into small pieces and transplanted to 20 petri dishes to obtain subcell strains.

Subsequent selection was repeated using the same procedure, but as selection advanced, the viability of the subcell strains became lower. Moreover, colonies isolated from the surviving subcell strains grew so slowly that they could be transferred only irregularly to liquid modified LS medium supplemented with 1 μM 2,4-D and 1 μM kinetin. We obtained 21 cell strains from the 40 substrains that were separable into two groups; 14 yellow-green fluorescent strains and 7 blue-white fluorescent strains, by repeating selection 6-13 times.

High producing cell lines for anthocyanin [7], shikonin [6], and berberine [8] have been established by cell aggregate cloning using the naked eye. However, we could not use this visual selection method because cultured *Rauwolfia serpentina* cells produce colorless alkaloids. Hence, we developed the selection method using UV-light for the detection of alkaloid fluorescence, and could select two groups of fluorescent cell suspen-

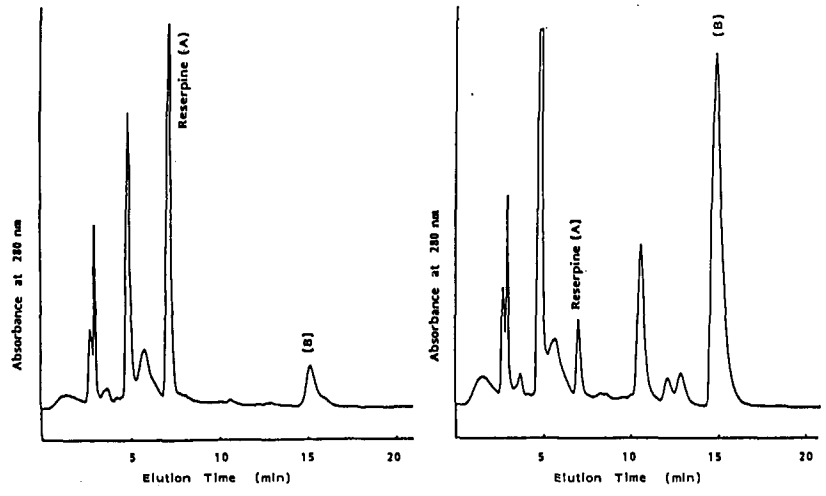

Figure 2. High performance liquid chromatograms of the MeOH extracts of yellow-green fluorescent cells (left) and blue-white fluorescent cells (right).

sion cultures from the heterogeneous callus cultures by cell aggregate cloning.

Correlation between reserpine content and fluorescence in selected strains

Seventeen of the twenty-one cell strains obtained by the fluorescence assay were subcultured for three generations under the same culture conditions to determine whether there was a correlation between their reserpine contents and fluorescence. We estimated the reserpine content by HPLC and the fluorescence by eye under 365 nm UV-light for 47 of the 51 suspension cultures. Fluorescence varied in the same group of fluorescent cell strains during incubation and could be classified into 5 colors; pale violet, blue-white, blue yellow-green, faint yellow-green, and yellow-green (Figure 1). Although reserpine showed considerable dispersion within the same fluorescent suspension culture, the yellow-green fluorescent cultures tended to produce more reserpine than the blue-white ones. This nonvisual selection, using the fluorescence of cell colonies as an indicator of alkaloid production, served as a rough but convenient and practical method for the qualitative estimation of reserpine.

Characteristics of the seconary selected strains and the optimum conditions for reserpine production

Previous investigation of the effects of plant hormones on growth and reserpine production in primary, selected suspension cells, showed that a hormonal combination of 1 μM 2,4-D and 0.1 μM kinetine or 10 μM NAA and 10 μM BA was more effective than that of 1 μM 2,4-D and 1 μM kinetin [10]. Therefore, we examined the effects of plant hormones as well

Table 1. Cell growth and reserpine and 3,4,5-trimethoxy benzoic acid contents in the different fluorescent cell strains.

Hormonal combination	Fluorescent cell strain	1st generation			2nd generation			3rd generation		
		Cell growth	Reserpine content	Trimethoxy benzoic acid content	Cell growth	Reserpine content	Trimethoxy benzoic acid content	Cell growth	Reserpine content	Trimethoxy benzoic acid content
		g fr. wt / flask	% of dry wt	% of dry wt	g fr. wt / flask	% of dry wt	% of dry wt	g fr. wt / flask	% of dry wt	% of dry wt
2,4-D 1 μM + kinetin 1 μM	Yellow-green	13.5	0.030	0.015	12.1	0.027	0.066	11.7	0.023	0.126
	Blue-white	12.5	0.0014	0.044	13.0	0.0016	0.083	13.1	0.0025	0.108
2,4-D 1 μM + kinetin 0.1 μM	Yellow-green	13.0	0.030	0.007	12.7	0.069	0.007	10.3	0.028	0.016
	Blue-white	12.4	0.0063	0.062	14.6	0.017	0.019	13.1	0.0022	0.061
NAA 10 μM + BA 10 μM	Yellow-green	11.9	0.109	0.014	13.0	0.101	0.021	11.2	0.143	0.164
	Blue-white	11.5	0.0019	0.123	13.4	0.0078	0.114	13.2	0.0141	0.062

Each value is the mean of 3 replicates.
Cell suspensions were cultured for 3 weeks in modified LS medium containing the different plant hormone concentrations given.
For measurement of fresh weight, cell suspensions were collected on a 62-μM nylon sieve.

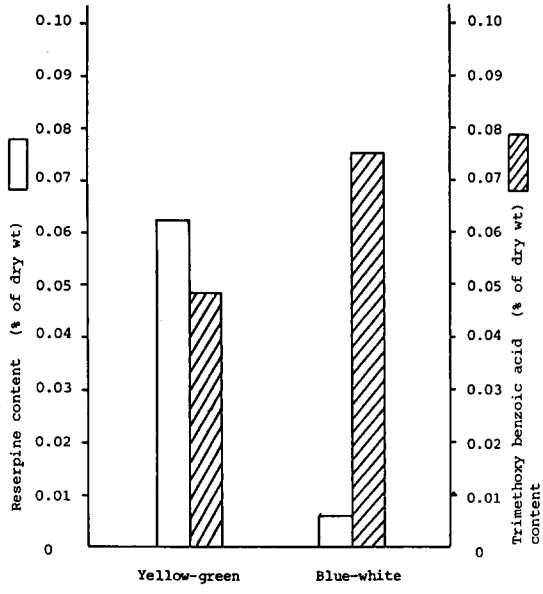

Figure 3. Correlation between reserpine and 3,4,5-trimethoxy benzoic acid contents in secondary, selected *Rauwolfia serpentina* cell strains.

as the optimum conditions for reserpine production in secondary, selected suspension cells that had been maintained for about 6 months without selection, using the same culture conditions. The yellow-green and blue-white fluorescent cell strains were the experimental materials used. These strains were subcultured three times in the dark for 3-week periods on modified LS medium containing different plant hormones. We found that product (B) might be related to reserpine (A) from the results of our HPLC analysis. Product (B) corresponded to an authentic sample of 3,4,5-trimethoxy benzoic acid. High performance liquid chromatograms of the MeOH extracts of both the yellow-green and blue-white fluorescent suspension cells are shown in Figure 2.

We know of no report of the biosynthesis of reserpine in cultured *Rauwolfia serpentina* cells. The biosynthesis sequence for rauwolfia alkaloid has been made clear by the isolation of alkaloidal components from cultured *Catharanthus roseus* cells [3,12]. It is suggested that reserpine also is synthesized from tryptamine and secologanin in cultured *Rauwolfia serpentina* cells. In the final step of reserpine biosynthesis, reserpine however might be produced by the condensation of methyl reserpate with 3,4,5-trimethoxy benzoic acid.

Cell growth and the reserpine and 3,4,5-trimethoxy benzoic acid contents of our different fluorescent cell strains are summarized in Table 1. We confirmed that all our selected strains maintain stable fluorescence as long as they are regularly subcultured.

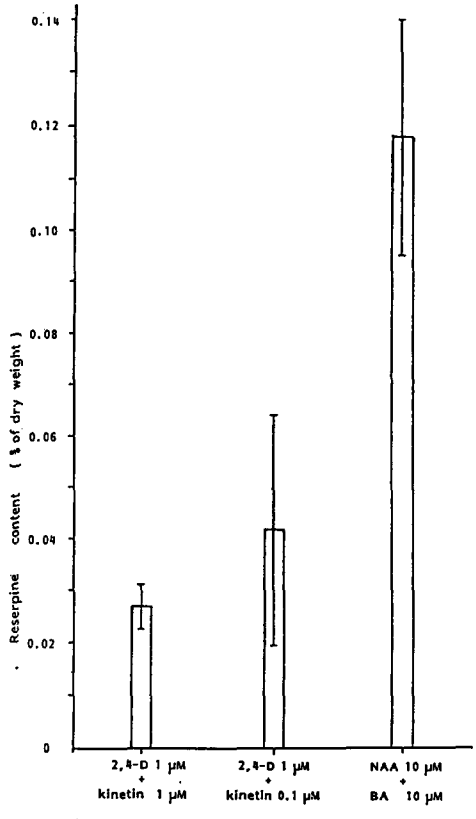

Figure 4. Effects of plant hormones on reserpine production in yellow-green fluorescent *Rauwolfia serpentina* strains. Bars represent standard deviations of the mean values.

We made a statistical analysis of the correlation between the reserpine and 3,4,5-trimethoxy benzoic acid contents in our fluorescent cell strains using the data shown in Table 1. The correlation is shown in Figure 3. The yellow-green fluorescent strains produced much reserpine, whereas the blue-white strain yielded much 3,4,5-trimethoxy benzoic acid. The correlation between the reserpine content of the yellow-green fluorescent cells and the hormonal combinations used is shown in Figure 4. A combination of 10 μM NAA and 10 μM BA was the most effective for reserpine production. With this combination we obtained a stable yellow-green fluorescent cell strain that gives a yield of reserpine of approximately 0.1% of its dry weight.

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