# 3. CELL AND TISSUE CULTURES OF *PERILLA*

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## INTRODUCTION

In recent years, plant cell and tissue culture has received a great deal of attention for the production of useful plant-specific chemicals. The advantages of plant cell culture compared with whole plant cultivation are that: cell cultures are unlimited by environmental, ecological or climate conditions; cells can proliferate at a higher growth rate; a large amount of cells can be obtained in a bioreactor with limited space; metabolite accumulation can be improved through regulation of culture conditions; downstream processing of products from cell cultures may be relatively easier.

The first report dealing with cell and tissue cultures of *Perilla* may be that by Sugisawa and Ohnishi (1976), and there have been many related publications since the 1980s. As shown in Table 1, these works were concerned with the formation of perilla pigments, caffeic acid, monoterpenes, sesquiterpenes, and essential oil by *Perilla* cells, as well as glucosylation, resolution, and morphogenesis of the cell cultures.

The cultural factors which affect cell growth and metabolite accumulation in plant cell cultures include biological (cell line, culture age, inoculum density, and cell aggregate size), chemical (such as medium composition), and physical factors (such as temperature, light irradiation, oxygen supply, and shear stress).

## CELL AND TISSUE CULTURES

### Callus Culture

#### *Basic requirements and media*

The basic requirements for plant tissue culture work are: (i) an area for medium preparation; (ii) a sterile room or sterile air cabinet for aseptic transfer; (iii) a constant temperature room or incubator for growth of callus cultures; (iv) shaker facilities for cell suspension cultures. The main physical requirement for growth and maintenance of plant cell cultures is constant temperature. Callus cultures are grown in plastic Petri dishes, glass culture tubes or plastic pots with screw cap lids. Suspension cultures are usually in glass conical flasks (Dixon, 1985).

Components of media for the growth of plant callus and suspension cultures can be classified into six groups, and this division is usually reflected in the way in which stock

**Table 1** Reports on cell and tissue cultures of *Perilla*

| | *Culture conditions* | *Authors (year)* |
|---|---|---|
| Metabolites: | | |
| perilla pigments | MS medium, 100 ppm NAA, 2 ppm KT, 25°C, with light | Ota (1986) |
| | LS medium, 10 μM NAA, 1 μM BA, 25°C, light 3000 lux for 12 h | Koda *et al.* (1992) |
| | LS medium, 1 μM 2,4-D and 1 μM BA, 25°C, Light at 17–20.4 W/m$^2$ | Zhong *et al.* (1991, 1993a, 1994a) |
| phenylpropanoids | $B_5$ medium, 5 ppm NAA, 1 ppm KT, 25°C, light at 2000 lux | Tamura *et al.* (1989) |
| caffeic acid | MS medium, 1 ppm 2,4-D, 0.1 ppm KT | Ishikura *et al.* (1983) |
| monoterpenes | MS medium, 1 ppm 2,4-D, 5 ppm KT 25°C, slightly dark | Sugisawa & Ohnishi (1976) |
| sesquiterpene | MS medium, I ppm NAA, 1 ppm KT, 25°C, light 3000 lux | Nabeta *et al.* (1985) |
| | modified MS, 1 ppm 2,4-D, 5 ppm KT | Shin (1986) |
| ursolic acid | LS medium, 1 μM NAA, 10 μM KT | Tomita & Ikeshiro (1994) |
| cuparene | MS medium, 1 ppm NAA, 1 ppm KT 25°C, light at 3000 lux | Nabeta *et al.* (1984) |
| essential oil | modified MS, 1 ppm NAA, 5 ppm KT 27±2°C | Shin (1985) |
| Glucosylation | LS medium, 1 μM 2,4-D, 25°C, dark | Tabata *et al.* (1988) |
| | MS medium, 1 μM 2,4-D, 26°C, dark | Furukubo *et al.* (1989) |
| Resolution | LS medium, 2,4-D, 26°C, dark | Terada *et al.* (1989) |
| Morphogenesis | MS medium, NAA, or 2,4-D, BA, NOA | Tanimoto & Harada (1980) |

Abbreviations: BA, benzylamino-purine; 2,4-D, 2,4-dichlorophenoxyacetic acid; KT, kinetin; NAA, 1-naphthaleneacetic acid; NOA, naphthoxyacetic acid. MS: Murashige and Skoog's, LS: Linsmaier and Skoog's.

solutions are prepared and stored. The groups are: (i) major inorganic nutrients; (ii) trace elements; (iii) iron source; (iv) organic supplement (vitamins); (v) carbon source; (vi) organic supplement (plant growth regulators). Table 2 shows a typical medium for *Perilla frutescens* cell cultures (Zhong *et al.*, 1991).

### *Callus induction*

The callus of *P. frutescens* was induced as follows: seeds were germinated on an agar medium of LS (Linsmaier and Skoog) minus growth regulators to produce young seedlings for use as a source of explants. Young leaf sections (5 mm$^2$) were excised and transferred to MS (Murashige and Skoog) basal medium containing sucrose (30 g/L), 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 pprn), kinetin (KT) (5.0 ppm) and Difco bacto-agar (0.9% w/v). The callus tissue was subcultured every 3 weeks during 6 months at 25°C in poor light conditions. A suspension culture derived from the callus of the

**Table 2** Medium composition for cell cultures of *P. frutescens*

| *Inorganic* | *Concentration (mg/L)* | *Organics* | *Concentration (mg/L)* |
|---|---|---|---|
| $KNO_3$ | 1900 | Myo-inositol | 100 |
| $NH_4NO_3$ | 1650 | Thiamine HCl | 0.4 |
| $CaCl_2 2H_2O$ | 440 | | |
| $MgSO4 7H_2O$ | 370 | Sugar | Concentration (g/L) |
| $KH_2PO_4$ | 170 | Sucrose | 30 |
| $Na_2$-EDTA | 37.3 | | |
| $FeSO_4 7H_2O$ | 27.8 | Hormone | Concentration (μM) |
| $MnSO_4 4H_2O$ | 22.3 | 2,4-D | 1 |
| $ZnSO_4 7H_2O$ | 8.6 | 6-BA | 1 |
| $H_3BO_3$ | 6.2 | | |
| KI | 0.83 | | |
| $Na_2MoO_4 2H_2O$ | 0.25 | pH | 5.8–6.0 (before autoclaving) |
| $CoCl_2 6H_2O$ | 0.025 | | |
| $CuSO_4 5H_2O$ | 0.025 | | |

eighth generation was maintained in a similar medium, without agar, on a rotary shaker at 25°C for 6 weeks (Sugisawa and Ohnishi, 1976).

### *Cell line selection*

Selection experiments have already yielded a large number of mutants. There are three approaches of cell line cloning: by plating cell aggregates, by protoplast culture, and by single cell culture. Selection of high-producing cell line by the method of cell aggregate cloning is as follows (Yamamoto *et al.*, 1982). The calli were cut into many segments (volume, *ca.* 3 $mm^3$) 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 the liquid medium and 0.8 % (w/v) agar. The segments were cultured under suitable conditions for a certain period. Each of the 9 segments on 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 (volume, *ca.* 3 $mm^3$). *All* these segments were coded and transplanted onto fresh medium in a 9-section Petri dish. This selection procedure was repeated many times.

## Suspension Culture

### *Flask culture*

Flask suspension cultures were obtained by the transfer of friable callus lumps to an agitated liquid medium of the same composition as that used for the growth of callus. A relatively large initial inoculum was advantageous, as this would ensure that sufficient single cells and/or small clumps were released into the medium to provide a suitably

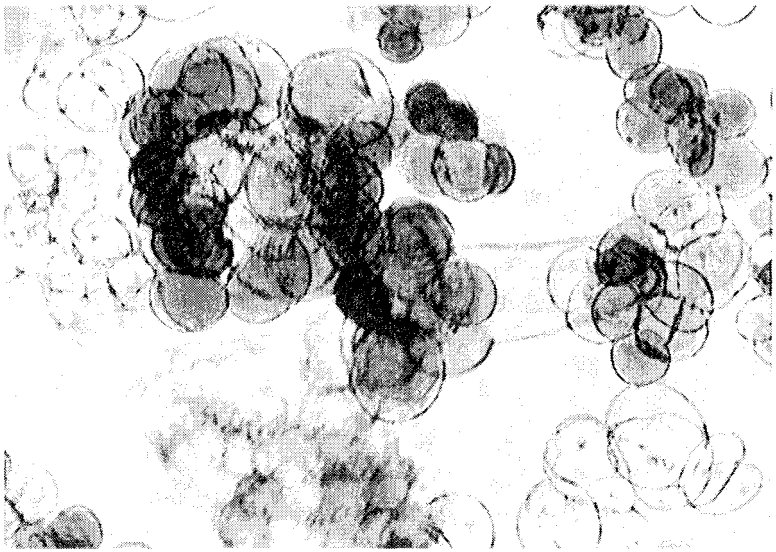

**Figure 1** Photograph of suspension cells of *Perilla frutescens* cultured in a shake flask

high cell density for subsequent growth. Rotational speeds of the orbital shakers should be in the range of 30–150 rpm with an orbital motion stroke of 2–4 cm.

For example, suspension cells of *P. frutescens* (Figure 1), which produced a high level of anthocyanin, were cultured in a 500 ml conical flask containing 100 ml LS medium with the addition of 3% sucrose, 1 μM 2,4-D and 1 μM benzylamino-purine (BA). The cells were incubated on a rotary shaker (75 rpm) at 25Â°under continuous light irradiation supplied by ordinary fluorescent lamps (17–20.4 $W/m^2$). The subculture period and inoculum density were generally controlled at 7–10 days and 25 g wet cells/L, respectively (Zhong *et al.*, 1991).

### *Bioreactor* culture

Large-scale culture of plant cells has developed from the need to study the problems of scale-up in the development of commercial processes for the production of biomass or secondary products. However, the growth of plant cell suspensions in bioreactors also allows the study of the effects of conditions such as aeration, oxygen and carbon dioxide levels on growth and secondary product formation, a study not possible in shake flasks.

For bioreactor operation, a reactor was filled with a certain amount of medium and autoclaved at 121°C for 30 min prior to the start of a cultivation. After the reactor had cooled to room temperature, the agitation speed and aeration rate were set as required. The cultivation temperature was set and then automatically controlled as required. Inoculation was performed by pouring the inoculum through a large opening in the head plate. The inoculum density was the same as in flask cultivation.

**Table 3** Pigment content of *P. frutescens* after cell line selection

| *Selection generation* | *3rd* | *4th* | *5th* |
|---|---|---|---|
| Pigment content (mg/g dry cell) | 80.6 | 196.4 | 197.1 |

## FACTORS AFFECTING METABOLITE FORMATION BY *PERILLA* CELLS

### Cell Line, Cell Aggregate Size, Subculture Period and Inoculum Density

#### Cell line

In the production of anthocyanins (pigment) and rosmarinic acid (phenylpropanoid) from cultured callus tissue of Akachirimen-shiso (*Perilla* sp.), Tamura *et al.* (1989) claimed that the relative amount of certain anthocyanins produced by the callus tissue was different and greater than found in the intact plant. However, by means of several subcultures, in which the pigmented cell line was selected, the anthocyanins of the callus were changed in amount and nature to that found in the intact plant. This change might be attributable to gene mutation in the cultured cell.

In anthocyanin production by *P. frutescens* cells, a high-pigment-producing strain in the cultured cells was selected through the method of cell-aggregate cloning as described above. The result is shown in Table 3 (Yoshida, M., Master thesis, Osaka Univ., 1989), in which the pigment content was measured as described elsewhere (Zhong *et al.*, 1991).

#### Cell aggregate *size*

The influence of the size of the cell-aggregate in suspension culture of *P. frutescens* on anthocyanin accumulation was investigated by inoculating with cell-aggregates of a screened size in successive subcultures, while for the control, the cell aggregate sizes were without screening. As shown in Table 4, compared with the control, the anthocyanin

**Table 4** Effect of sizes of cell aggregates on anthocyanin accumulation by *P. frutescens* cells subcultured successively in a shake flask'

| *Size* (μm) | *Anthocyanin content (mg/g dry cell)* *Subculture* | | | | |
|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th |
| Control | 90 | 87.4 | 81.7 | 90.2 | 77.9 |
| 250-2000 | 67.6 | 56.2 | 66.2 | 54.5 | 42.1 |
| 149-250 | 76.3 | 50.3 | 81.8 | 79.7 | 81.4 |
| 37-149 | 105 | 71.9 | 88.7 | 85.5 | 64.9 |

'The cell growth was almost the same in all the above cases. The data shown here was an average of at least 3 samples. The cell aggregate sizes of the control were in the range of 37-2000 μm with a distribution similar to that as described (Zhong *et al.*, 1992).

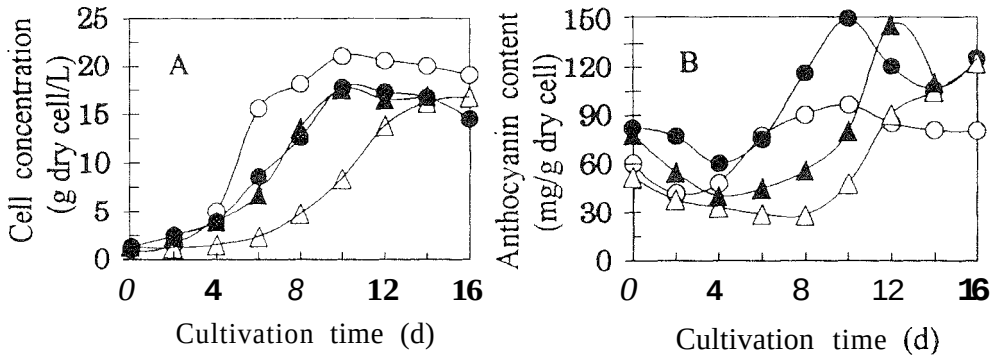


Figure 2 Effect of subculture period on anthocyanin accumulation by *P. frutescens* cells in a flask culture. Symbols for subculture period: Open circle, 5 d; closed circle, 7 d; closed triangle, 10 d; open triangle, 14 d

content of flask cultures which were inoculated with cell aggregate sizes greater than 250 μm was lower. The pigment content in other cultures, which were inoculated with cell aggregate sizes smaller than 250 μm, was almost the same as that of the control. The result suggests that cell-to-cell communication, which may be important to the metabolite formation, probably depends on a certain range of cell aggregate sizes.

*Subculture period*

The effect of subculture periods on *P. frutescens* cell cultures was studied by subculturing the cells at an interval of 5, 7, 10 or 14 days (Zhong and Yoshida, 1994d). The results indicated that the cells subcultured at an interval of 7 or 10 days yielded a higher anthocyanin content, i.e. higher specific anthocyanin production (mg/g dry cells), compared with those at an interval of 5 or 14 days. In these cases, the anthocyanin content proved to be unstable during subcultures. The results indicated that alteration of the subculture period is one way of overcoming the instability of metabolite production in plant cell cultures, which is one of the main obstacles to the commercialisation of plant cell culture processes.

A further investigation on the growth and production dynamics of the cell cultures at different subculture periods indicated that although the cells which were subcultured at an interval of 5 days propagated relatively faster, the anthocyanin content was rather lower, compared with those at an interval of 7 and 10 days. The cells subcultured at a 14-day interval showed lower cell growth and anthocyanin content, compared with those at a 7- or 10-day interval (Figure 2). The results indicate that the physiological and metabolic aspects of cultured cells varied greatly with different subculture periods.

### *Inoculum density*

The effect of inoculum density (15, 25, 50 and 75 g wet cells/L) on cell growth and anthocyanin formation by suspension cells of *P. frutescens* was investigated by using a 500 ml conical flask containing 100 ml medium (Zhong and Yoshida, 1995). The results showed that the growth rate and cell concentration reached at around 10th day were quite similar for different inoculum densities, and the substrate (sugar) was also almost completely consumed by that day in all the cases (Zhong and Yoshida, 1995). However, the anthocyanin content (i.e. specific anthocyanin production, mg/g dry cells) and total anthocyanin production (g/L) were very different at various inoculum densities. The production and specific production of the pigment reached the highest at an inoculum density of 50 g wet cells/L (Zhong and Yoshida, 1995).

## Chemicals

### *Carbon source*

In plant cell cultures, sucrose is usually a suitable carbon source. In *P. frutescens* cultures, Harada (Harada, H., Master thesis, Osaka Univ., 1988) and Koda *et al.* (1992) found that among different carbon sources, sucrose was found to be the best one for both the cell growth and formation of anthocyanin pigment.

In high density batch cultures of P. *frutescens* (at an inoculum size of 50 g wet cells/L), the initial sucrose concentration showed a conspicuous effect on the kinetics of cell growth, sugar consumption, and anthocyanin production by P. *frutescens* cells. The maximum cell density of 38.3 g dry cells/L was obtained after 11 days' cultivation at an initial sucrose concentration of 60 g/L, while the highest pigment production of more than 5.8 g/L was attained at 45 g/L of sucrose (Zhong and Yoshida, 1995).

In addition, the data indicated that the initial sucrose concentration affected the excretion of anthocyanin by cell cultures of P. *frutescens* (Zhong *et al.*, 1994c). When the medium contained 40–50 g/L of sucrose a much higher amount of anthocyanin was released from the cultured cells, compared with the control of 30 g/L of sucrose. Further experiments confirmed this result.

### *Nitrogen source*

An investigation of the effect of the nitrogen source on the growth and anthocyanin production by *P. frutescens* cells has been carried out. The results indicated that the total amount of nitrogen and the ratio of nitrate to ammonium salts in the LS medium were the most suitable for the cell cultures (Harada, H., Master thesis, Osaka Univ., 1988). But a study made by another group showed that a $NO_3^-/NH_4^+$ of 10 with a total nitrogen of 30 mM gave the best results for the growth of, and pigment formation by, *P. frutescens* cultures (Koda *et al.*, 1992).

### *Plant growth regulator*

Table 5 shows that caffeic acid of 1.1–2.7 mg per g fresh weight of calluses were estimated to be present in *Perilla* calli. The yield from the cells in MS-III medium containing

**Table 5** Caffeic acid formation in *Perilla* callus cultured in various media

| Agar media | Growth regulators (mg/L) | | | Fresh wt. (g) of callus subcultured | Fresh wt. (g) of callus cultured for 21 days | Caffeic acid (mg) per g fresh wt. of the callus |
|---|---|---|---|---|---|---|
| | 2,4-D | NAA | kinetin | | | |
| SH-M | 0.5 | — | 0.01 | 3.89 | 18.32 | 1.82 |
| MS-I | 1.0 | — | 0.1 | 3.64 | 20.00 | 1.53 |
| MS-II | 1.0 | — | 5.0 | 4.15 | 20.18 | 2.67 |
| MS-III | — | 1.0 | 0.1 | 4.01 | 12.11 | 2.60 |
| G | 1.0 | — | — | 3.85 | 19.45 | 1.12 |

1-naphthylacetic acid (NAA, 1.0 mg/L) instead of 2,4-D and in MS-II medium containing a high concentration of kinetin (5.0 mg/L) were about double those in MS-I and G media (Ishikura *et al.*, 1983).

Shin (1985) reported that an addition of one ppm of NAA instead of 2,4-D in the modified MS medium containing 5 ppm of kinetin caused 75% increase in the growth of *P. frutescens* cells and two-fold increase in production of essential oils.

In anthocyanin production, Harada's result indicated that 1 μM of 2,4-D in combination with 1–10 μM of BA in LS medium was the most suitable for cell growth and pigment formation (Harada, H., Master thesis, Osaka Univ., 1988) whereas Koda *et al.* (1992) claimed that 10 μM NAA in combination with 1 μm BA was the best for P. *frutescens* cultures (Table 6).

*Precursor*

In a two stage culture of the young leaf of *Perilla* species, Shin (1986) reported that the addition of mevalonic acid lactone (10 ppm) increased the wet cells and yield of essential oil (including sesquiterpene hydrocarbons and sesquiterpene alcohol) of callus from 1.8 to 2.6 g and from 4.7 to 18.7 mg/tube, respectively.

**Table** 6 Effects of phytohormones on growth and pigment production by *P. frutescens* cells

| Auxin | Cytokinin | Fresh wt. (g/flask) | Pigment (CV/g) | Pigment (CV/flask) |
|---|---|---|---|---|
| 2,4-D $10^{-5}$ M | BA $10^{-6}$ M | 3.80 | 0.66 | 2.51 |
| IAA | | 1.09 | 1.54 | 1.68 |
| NOP | | 3.21 | 4.25 | 13.64 |
| NAA $10^{-4}$ M | | 2.81 | 3.87 | 10.87 |
| NAA $10^{-5}$ M | BA $10^{-5}$ M | 3.52 | 6.12 | 21.54 |
| | BA $10^{-6}$ M | 3.21 | 6.84 | 21.96 |
| | BA $10^{-7}$ M | 1.85 | 4.84 | 8.95 |
| NAA $10^{-6}$ M | BA $10^{-6}$ M | 1.99 | 3.15 | 6.27 |

CV = $[OD_{524}$ (Sample wt (g) + 10)]/Sample wt (g)
Abbreviations: BA, benzylamino-purine; 2,4 D, 2,4-dichlorophenoxyacetic acid, IAA, indole-3-acetic acid; NAA, 1-naphthaleneacetic acid, NOP, naphthoxypropionic acid.

**Table 7** Growth and volatile matter content in *Perilla* callus fed with and without mevalonate

| | *Without* | *With mevalonate* | | | |
|---|---|---|---|---|---|
| | | *Non-labeled* | *2,2-$^2H_2$-* | *4,4-$^2H_2$* | *5,5-$^2H_2$* |
| Growth index | 5.87 | 1.29 | 1.29 | 1.29 | 1.06 |
| Volatile content ($10^{-3}$% fresh wt.) | 0.067 | 1.83 | — | — | — |
| Sesquiterpene content ($10^{-3}$% fresh wt.) | 0.013 | 0.025 | — | — | – + |
| Sesquiterpenes | | | | | |
| Isolongifolene | + | | + | | + |
| α-Curcumene | + | + | | + | |
| β-Bisabolene | + | + | + | + | + |
| Chamigrene | + | | + | | + |
| Thujopsene | + | + | | | + |
| Cuparene | + | + | + | + | + |
| α-Farnesene | + | + | | | |
| β-Farnesene | + | + | | | |
| Farnesol | + | + | | | |

, not determined; +, detected by GC/MS analysis.

Later, Nabeta *et al.* (1993) claimed that the administration of mevalonate (MVA, 10 mmol/8 ml culture medium) significantly retarded the growth of *Perilla* callus tissues [growth index (fresh wt.): 1.06–1.29 with MVA and 5.87 without, see Table 7]. However, a significant increase in the level of total volatiles was observed (24 times higher than that in the calli grown without MVA, see Table 7). The increase in the total volatile content was mainly due to the formation of long-chain compounds including palmitic acid, tetradecane, 2,4-decadienal and butyric acid. The level of sesquiterpene hydrocarbons in the calli with MVA was 1.8 times higher than that in the calli without. Sesquiterpene hydrocarbons were always observed in the calli cultured with the deuterated MVAs, and deviations in their qualitative patterns were observed upon the addition of the differently deuterated MVAs. Cuparene and β-bisabolene, however, were always observed.

*Inhibitor*

In order to clarify the biosynthetic regulation of caffeic acid in *Perilla* cell suspension cultures, Ishikura *et al.* (1983) examined the response of the cells to three inhibitors, L-2-aminooxy-3-phenylpropionic acid (L-AOPP), 2-aminooxyacetic acid (AOA) and N-(phosphonomethyl) glycine (glyphosate). The administration of L-AOPP, AOA and glyphosate to the *Perilla* cells inhibited caffeic acid formation to a large extent. An 80% inhibition of caffeic acid formation was caused by $10^{-4}$ M L-AOPP whereas phenylalanine and tyrosine contents of the cells became 7.5 and 2.3 times higher at this L-AOPP concentration than those in the control. An 85% inhibition of caffeic acid formation was achieved at $10^{-3}$ M glyphosate concentration, while $10^{-3}$ M AOA inhibited caffeic acid formation by 95% and also growth rate by 80%.

**Table** 8 Comparison of parameter values in cultivation of *P. frutescens* cells at different temperatures in flask cultures

| $T$ (°C) | $\mu$ ($d^{-1}$) | $X_{max}$ (g/L) | $AC_{max}$ (mg/g) | $TA$ (g/L) | $Y_{X/S}$ (g/g) | $Y_{P/S}$ (g/g) | $Q$ (mg/L/d) |
|---|---|---|---|---|---|---|---|
| 22 | 0.21 | 21.6 | 185.1 | 3.67 | 0.70 | 0.115 | 211 |
| 25 | 0.32 | 19.9 | 176.9 | 3.52 | 0.66 | 0.112 | 268 |
| 28 | 0.37 | 19.2 | 67.6 | 1.25 | 0.62 | 0.032 | 68 |

T, temperature; $\mu$, specific growth rate; $X_{max}$, maximum cell concentration; $AC_{max}$, maximum anthocyanin content; TA, total anthocyanin; $Y_{X/S}$, cell yield; $Y_{P/S}$, anthocyanin yield; Q, volumetric anthocyanin productivity.

In a related work, Ishikura and Takeshima (1984) reported that when 1 mM glyphosate was added to the cell culture in the logarithmic and stationary phases, the amount of caffeic acid ceased to increase and remained at a nearly constant level during the following several days. The shikimic acid content of cells from 14-day culture grown in the presence of 1 mM glyphosate increased up to 74.9 μg per g fresh weight during 6-day culture, whereas that of the control cells was undetectable. The dosage of 0.15 mM L-AOPP, an inhibitor of phenylalanine ammonia-lyase, to the cells did not cause shikimic acid accumulation.

### Temperature

The effect of culture temperature on cell growth and anthocyanin formation by the cell cultures of *P. frutescens* has been investigated (Zhong and Yoshida, 1993b). The results showed that at different incubation temperatures (i.e. 22°C, 25°C and 28°C), although the maximum cell concentration obtained was identical (about 20 g/L), the growth rate, anthocyanin content and total anthocyanin produced were very different. At a higher temperature, in a range of 22~28°C, a higher specific growth rate was obtained. However, anthocyanin production, its productivity and yield were remarkably reduced at 28°C compared with those at 22°C and 25°C, respectively (Table 8). The highest anthocyanin productivity was obtained at 25°C. The above results indicated that the culture temperature was an important factor regulating the mechanism of anthocyanin biosynthesis in cultured cells of *P. frutescens*, and that this parameter should be strictly controlled during these cultivations.

### **Light** Irradiation

In plant cell-tissue cultures, light has stimulatory, inhibitory, or insignificant effects on cell growth and the accumulation of plant metabolites. For example, in modified MS medium containing 1 ppm of 2,4-D and 5 ppm of kinetin, Shin (1985) claimed that light (irradiation at 1600 lux) decreased the callus growth by 25% but rather increased the content of essential oil by two-fold, i.e. from 2% to 4%.

Table 9 Effect of light source on cell growth and anthocyanin content of *P. frutescens* cells cultivated for 12 days in 500 ml conical flasks

| *Light spectrum* | *Growth* (*g wet cells/100 mL*) | *Anthocyanin content* (*mg/g dry cells*) |
|---|---|---|
| Ordinary fluorescent lamp | 27.3 | 296.1 |
| Plant lamp | 29.8 | 239.7 |
| UV lamp | 16.6 | 187.2 |

*The ordinary fluorescent lamp has light spectrum range of 450–610 nm; the plant lamp possesses two peaks in its light spectrum at 460 nm (blue) and 655 nm (red) for each; the UV lamp has a light spectrum peak at 325 nm. Light irradiation intensity was 1000–1100 lux in all the cases.

Until now, detailed investigations on the optimisation of light irradiation conditions are very scarce. More seriously, there is a lack of information concerning the optimisation of light irradiation on a bioreactor scale, although such an investigation is essential to the design and optimisation of large scale processes for metabolite production by plant cell cultures. Here, a systematic study on the effect of light irradiation on anthocyanin production by *P. frutescens* cells is described.

### *Influence of light spectrum on anthocyanin accumulation*

We found (Table 9) that irradiation from the light source of an ordinary fluorescent lamp with a spectrum range of 450–610 nm was most effective for anthocyanin production by *Perilla* cells, while irradiation by a plant lamp decreased the anthocyanin content a little, and irradiation by a UV lamp showed inhibition to both growth and pigment formation of the cultured cells. In other studies, Ota (1986) claimed that perilla pigment was produced by exposing to a 450–500 nm bluish fluorescent lamp, while no pigment production occurred with a 580 nm daylight fluorescent lamp.

### *Effects of irradiation period and light intensify on anthocyanin formation in a flask or roux bottle*

The effect of light irradiation period on anthocyanin content of *P. frutescens* cells was investigated (Zhong eta!., 1991). Although the cell mass was increased 12 fold compared with the inoculum in all the cases, the anthocyanin content of the cells was very different for each case. Light irradiation for the first 7 days or the whole cultivation period was effective for anthocyanin production, while only a small amount of anthocyanin was obtained in the cases of no light irradiation in the first 7 days, even when light was supplied in the subsequent 7 days.

Investigation of the effect of light intensity on pigment production was attempted by using a 500 ml roux bottle with a lighting area of 164 $cm^2$ at 0.4 vvm (Zhong *et al.*, 1991). The results demonstrated that after 14 days' cultivation the anthocyanin content and total production increased with the increase of light intensity up to 27.2 $W/m^2$ of the daylight irradiation supplied by an ordinary fluorescent lamp.

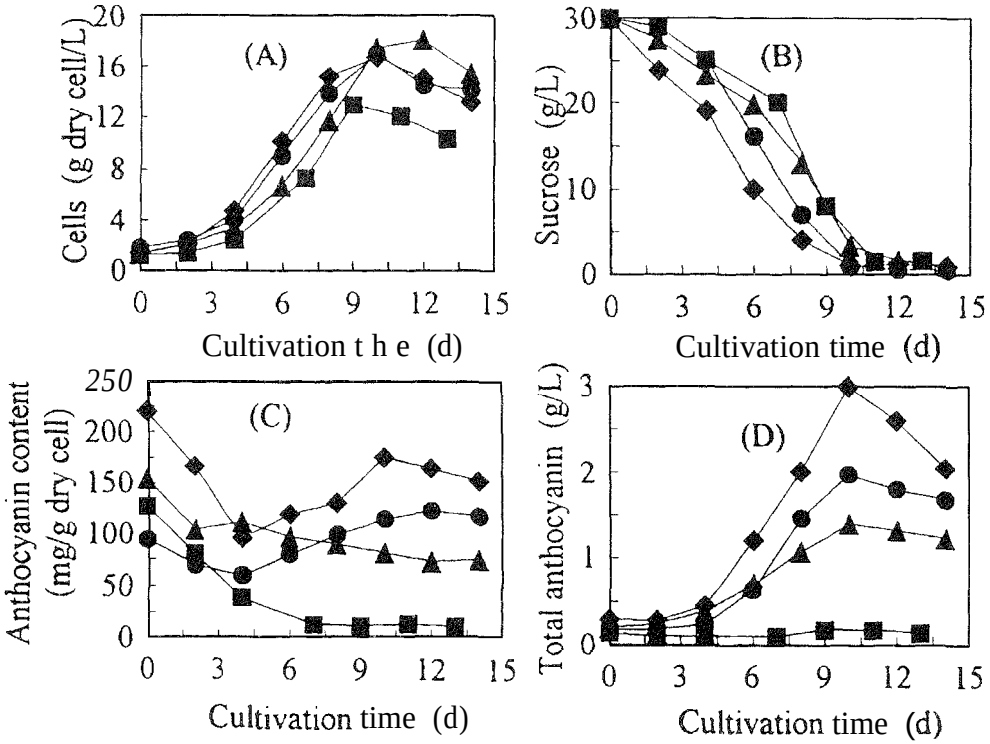


**Figure 3** Effect of light irradiation on anthocyanin production in cell cultures of *P. frutescens* in a 2-L (working volume) bubble column reactor. Symbols for light irradiation intensity ($W/cm^2$): triangle, 0; circle, 13.6; rhombus, 27.2; square, 54.4

*Effect of light irradiation on anthocyanin production in a bioreactor*

From the above results, to translate the small scale result to a bioreactor scale, it was clearly necessary to investigate what light irradiation intensity was most suitable for anthocyanin production by cultured cells of P. *frutescens* on the bioreactor scale. Experiments in a 2-L (working volume) bubble column reactor were made with light irradiation at different intensities while maintaining the other cultivation conditions the same. Figure 3 indicates that in the bioreactor cultivation without light irradiation, the total anthocyanin was 1.4 g/L. In a cultivation performed at 13.6 $W/m^2$ of light irradiation intensity, on the 10th day of the cultivation about 2 g/L of total anthocyanin was accumulated. When the light irradiation intensity was increased up to 27.2 $W/m^2$, a maximum anthocyanin of 3.0 g/L was obtained on the 10th day of the cultivation and this value is comparable to that accumulated in the flask culture where 3.5 g/L of total anthocyanin was formed after 12 days incubation (Zhong *et al.*, 1991). The pigment productivity (g/L/day) reached 2.5 and 1.5 fold compared with that without lighting or

with lighting at 13.6 W/m$^2$, respectively (Figure 3), and it was even a little higher than that in a flask culture. With a further increase of lighting intensity to 54.4 W/m$^2$, however, the total anthocyanin was markedly reduced (Figure 3). This means that such a high irradiation intensity was harmful to pigment formation. The results obtained above may serve as a guide to the optimisation of light irradiation in the scale up of the cultivation process for mass production of the anthocyanin.

## Oxygen Supply

Figure 4A shows a typical example of the time course of a cultivation in a 2-L turbine reactor (at 150 rpm and 0.2 wm). The maximum amount of anthocyanin produced was 0.48 g/L on day 11. The highest cell concentration, about 17 g dry cell/L, was also reached on that day. The anthocyanin content continuously decreased from 65 mg/g dry cell at the beginning to 25 mg/g dry cell at the end of cultivation.

The reasons why anthocyanin yield was poor under the above conditions had to be elucidated. Because the initial volumetric oxygen transfer coefficient ($k_La$) value was low (ca. 6.8 h$^{-1}$) (Zhong *et al.*, 1993a), it was considered possible to enhance pigment formation by improving oxygen supply to obtain results like those in cultivations using a shake flask (Zhong *et al.*, 1993a). Experiments were made by using a modified reactor with a sintered sparger to observe the effect of oxygen supply on anthocyanin production by the cells. In the modified reactor, at 150 rpm initial $k_La$ values of 9.9 and 15.2 h$^{-1}$ were obtained at 0.1 and 0.2 vvm, respectively (Zhong *et al.*, 1993a).

Figure 4B shows that the total amount of anthocyanin produced at aeration rates of 0.1 and 0.2 vvm was 0.55 g/L on day 10 and 1.65 g/L on day 12, respectively, while cell

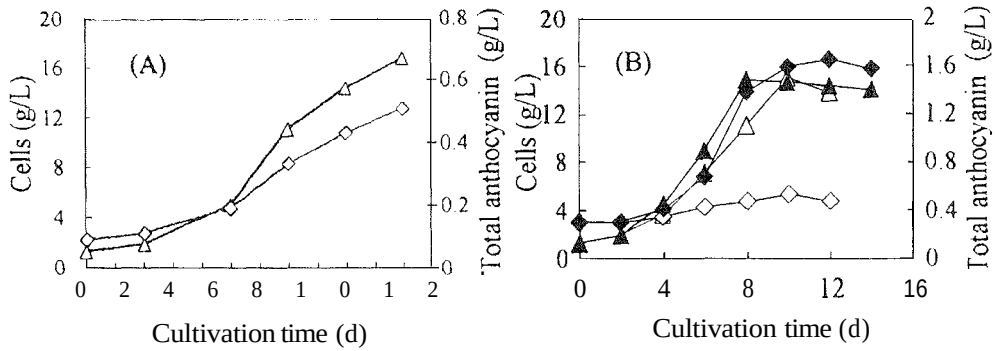


**Figure 4** Effect of oxygen supply on anthocyanin production in cell cultures of *P. frutescens* in a 2-L (working volume) stirred bioreactor at 150 rpm. A, ring sparger (at 0.2 wm); B, sintered sparger (at 0.1 and 0.2 vvm). Symbols: triangle, cell concentration; rhombus, anthocyanin production. In Figure 4B, white and dark symbols are for an aeration rate of 0.1 and 0.2 wm, respectively.

growth was virtually the same for both cases. Anthocyanin content decreased continuously from 250 mg/g dry cell to 35 mg/g dry cell on day 10 at a 0.1 vvm aeration rate. At an aeration rate of 0.2 vvm, the pigment content decreased from 220 mg/g dry cell to 75 mg/g dry cell on day 6, but after that increased to 120 mg/g dry cell on day 12. At an aeration rate of 0.1 vvm using the sintered sparger, the production of anthocyanin was poor, showing almost the same result as that at 0.2 vvm using a ring sparger (Figure 4A), when the other cultivation conditions were maintained the same. However, when the aeration rate was increased to 0.2 vvm with the new sparger being used, product accumulation increased almost 3-fold, i.e. 1.65 g/L was obtained compared to that at 0.1 vvm using the same sintered sparger or at 0.2 w m with the ring sparger. The results indicate that the oxygen supply conditions affected product biosynthesis.

In addition, regardless of the different cultivation conditions mentioned above, we observed a similar pattern of change in dissolved oxygen (DO) level during cultivation, with the lowest DO value being around 10–20% of air saturation. An investigation of the effect of D O level on anthocyanin production indicated that there was no difference in production at a D O level controlled at 20% or 80% of saturation (unpublished results). It is suggested that the phenomenon observed was due to differences in the oxygen uptake rate (OUR) between cells producing anthocyanin and those not producing anthocyanin, with the OUR of the former being larger. We confirmed such a difference in OUR between more anthocyanin-producing cells and less anthocyanin-producing cells (Zhong *et al.*, 1994b).

## Shear Stress

Plant cells are usually sensitive to hydrodynamic stress as each usually has a large volume (ranging from 20 to as much as 100 μm in diameter) and a rigid cellulose-based inflexible cell wall, and often has a very large vacuole, comprising up to 95 % or more of the cell volume. Such characteristics of plant cells imply that they are easily susceptible to damage under a certain degree of shear. In our case, the detrimental effects of shear stress on *P. frutescens* cells were demonstrated in both short-term experiments and batch cultivations in bioreactors (Zhong *et al.*, 1993a and 1994a).

The quantitative shear effects on cell growth and anthocyanin production by the cell culture were analysed in a series of batch cultures in a 3-L (working volume) plant cell reactor with a marine impeller having a diameter of 85 mm (larger impeller) or 65 mm (smaller impeller). Figure 5 shows the effects of the average and maximum shear rates on the specific growth rate and specific production of anthocyanin (i.e. anthocyanin content) of *P. frutescens* cells in the bioreactor cultivations. Here, the maximum shear rate is assumed to be represented by the impeller tip speed (ITS). The specific growth rate was apparently reduced at an average shear rate (ASR) over 30 $s^{-1}$ or at an ITS of over 8 dm/s. The anthocyanin content was relatively high at an ASR below 30 $s^{-1}$ or an ITS below 8 dm/s. At higher shear rates, the pigment content of the cultured cells showed an obvious decrease. The effects of shear on the maximum cell concentration, total anthocyanin production and the volumetric productivity of anthocyanin were similar to those as shown in this figure. Relatively high cell concentration, pigment production and pigment productivity, as well as cell and pigment yields, were achieved in the bioreactor

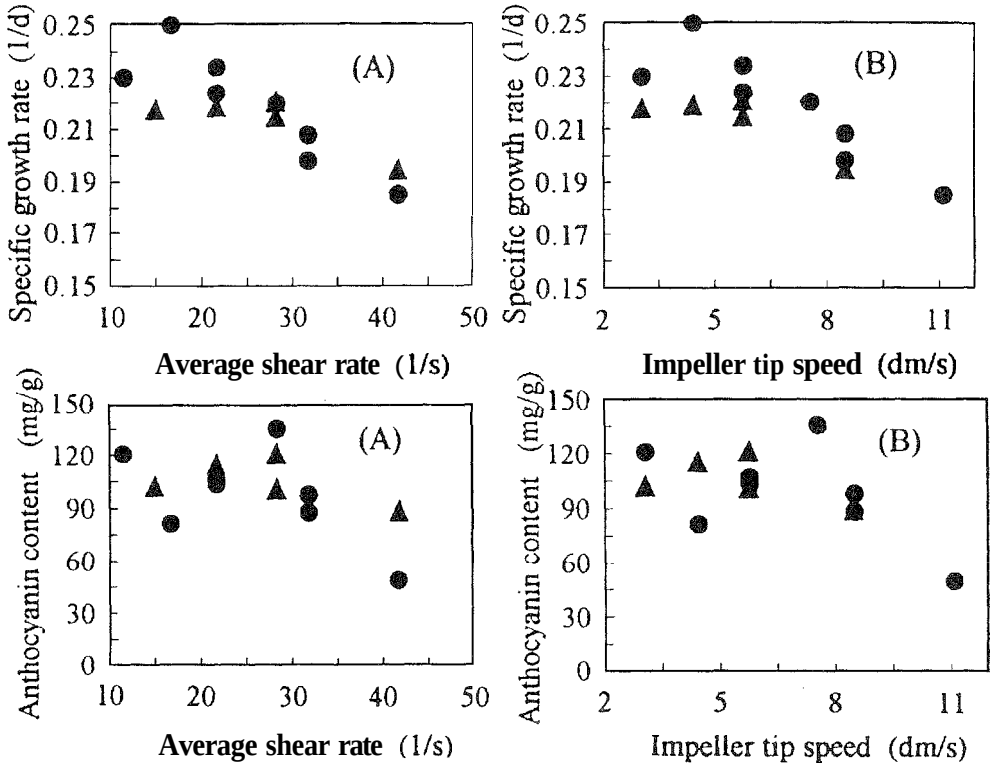


**Figure 5** Effect of shear on the specific growth rate and anthocyanin content in cell cultures of *P. frutescens* in a 3-L (working volume) plant cell bioreactor with a marine impeller. A, average shear rate; B, impeller tip speed. Symbols: circle, big impeller; triangle, small impeller

at an ASR of 20–30 $s^{-1}$ or at an ITS of 5–8 dm/s (Zhong *et al.*, 1994a). These criteria were equivalent to the following operational conditions in the reactor: an agitation speed of 120–170 rpm using the larger impeller (85 mm in diameter). At an ASR of over 30 $s^{-1}$ or an ITS of over 8 dm/s, the cell growth, pigment production and its specific production, as well as the yields of the cells and the pigments were poor due to the detrimental effects of the shear stress on the cultured cells.

## CONCLUSIONS AND INDUSTRIAL PERSPECTIVES

In this chapter, the basic techniques for cell and tissue cultures of *Perilla*, including callus induction, cell line selection as well as establishment of suspension cultures, were introduced; and the progress in the cell and tissue cultures since the 1970s has been

reviewed. The factors affecting the cell and tissue cultures of *Perilla* were cell line, cell aggregate size, inoculum density, culture age (biological factors); light irradiation, oxygen supply, shear stress, culture temperature (physical factors); precursor, inhibitor, and medium components (including carbon and nitrogen sources as well as plant growth regulators) (chemical factors). A systematic study on the optimisation of these factors for anthocyanin production was shown.

As demonstrated in our previous work (Zhong *et al.*, 1991), the bioprocess of anthocyanin production by suspension cells of *P. frutescens* was successfully scaled up from a shake flask to a bioreactor scale (with working volume of 3 L) by paying attention to the factors of light irradiation, oxygen supply and shear stress. Through a similar approach, the cell cultivation in a 100-L jar fermentor was succeeded by San-ei Chemical Industry Co. (Osaka, Japan) (Koda *et a!.*, 1992); the red pigment produced in the big fermentor was 7-fold increase compared with its inoculation level, and the amount was estimated to be equivalent to that extracted from 35 kg of fresh perilla leaves (Koda *et al.*, 1992). For commercial production of anthocyanin pigment by the cell cultures, however, much further improvement in the efficiency of the San-ei process was necessary.

In the case of famous shikonin process developed by Mitsui Petrochemicals Co., the shikonin productivity by cell cultures and the market value of the product in 1987 were 0.1 g/L/d and US$ 4000/kg, respectively (Ilker, 1987). In contrast, at that time the pure anthocyanin was sold at US$ 1250~2000/kg, while the highest pigment productivity obtained via cell culture was only 55 mg/L/d (Yamakawa *et al.*, 1983). Due to the relatively cheaper value and lower yield of anthocyanin compared with those of shikonin, it is apparent that further technological improvements were required for its practical industrial exploitation. In our recent work, a high anthocyanin productivity of ca. 0.3 g/L/d was achieved (in a bioreactor) (Zhong *et a!.*, 1991), and more recently it was further enhanced up to 0.58 g/L/d (in a shake flask) (Zhong and Yoshida, 1995). Here, it may be reasonable to consider that a big step has been taken towards the commercial anthocyanin production by cell cultures. Future work includes further bioreactor scale-up as well as the product safety test (especially for its application in food industries).

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