STUDIES ON PRODUCTION OF ANTHOCYANINS BY STRAWBERRY CULTURED CELLS

（イチゴ培養細胞によるアントシアニン生産に関する研究）

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CONTENTS

PREFACE

Anthocyanins, one of the major group of pigments, are responsible for the orange, red and blue colors in fruits, vegetables, flowers and other storage tissues in plants. Since many synthetic food colorants, particularly red ones, have been banned for use in food products due to their toxicity (Timberlake, 1986), various types of research on food colorants from natural sources have been carried out. The naturally occurring anthocyanin pigments have been considered as an alternative to synthetic red coloring agents.

Recently, investigations on anthocyanins have revealed that they have significant pharmacological effects on living organisms. Cyanidin-3-diglucoside-5-monoglucosiode, found in the red turnip, *Brassica campestris* L., is capable of reducing the atherogenic index in rats (Igarashi *et al.*, 1990). It was also reported that malvidin-3,5-diglucoside, found in wild grapes, significantly lowered the triglyceride and free fatty acid levels, and that the total cholesterol level in rats serum was markedly lower than that measured in control (Igarashi and Inagaki, 1991). Moreover, Kamei *et al.* (1993) reported that anthocyanins inhibit the growth of cancer cells. Studies concerning anthocyanins have therefore become very important recently not only for use as food additives but also in the field of pharmacology.

With recent advances in plant biotechnology, obtaining useful secondary metabolites in large quantities throughout all seasons became easy by using tissue cultures. There have been a number of reports on the production of secondary metabolites such as alkaloids, terpenes, quinones and flavonoids with plant tissue cultures. Anthocyanins have also been produced in various plant tissue cultures: wild carrot (Dougall and Weyrauch, 1980; Kinnersley and Dougall, 1980; Ozeki and Komamine, 1981), *Catharanthus roseus* (Carew, 1976), *Euphorbia millii* (Yamamoto *et al.*, 1982), *Petunia Hybrida* (Colijn *et al.*, 1981) *Strobilanthes dyeriana* (Smith *et al.*, 1981), *Vitis hybrida* (Yamakawa *et al.*, 1983a,b), *Hibiscus sabdariffa* (Mizukami *et al.*, 1988), *Populus nigra*

x *P.maximiwiczii* (Matsumoto *et al.*, 1970), *Vitis* sp (Tamura *et al.*, 1989) and strawberry (Hong *et al.*, 1989a).

Furthermore, studies on anthocyanin production are focused not only on improving productivity for large-scale production, but also on factors influencing anthocyanin accumulation. Such factors are UV (Wellmann *et al.*, 1976), light (Takeda, 1988), nitrogen sources (Do and Cormier, 1991a,b), types of sugars (Nakajima *et al.*, 1989), osmotic stress (Do and Cormier, 1991) and elicitors (Schnitzler *et al.*, 1989).

On the other hand, the strawberry is one of the most popular fruits in the world owing to its flavor and aroma. Although strawberries are eaten and enjoyed per se, the recovery of flavor concentrates, which would be of value to flavorists or food processors, is limited. Strawberry flavor concentrates and strawberry essence are used extensively in food industry to enhance the flavor of syrups, beverages, canned fruit mixtures, ice cream, frozen desserts, dry cake mixes, frosting and confectionery products. They are also used as flavoring agents for oral medications and vitamins. Therefore, study of tissue cultures regarding the environmental factors that influence the cell growth and pigment production of strawberry plants are important to develop their potential uses as a food ingredient or as a source of strawberry flavor. However, the studies of several researchers (Nitsch 1950, 1955; Adams 1972; Boxus 1974; Lee and de Fossad 1977), have mostly been concerned with micropropagation of meristem cultures for regeneration of strawberry plants.

Recently, Hong *et al.* (1989a) reported anthocyanin production in suspended cultures of immature strawberry fruits. They revealed that cultivars had different abilities to initiate callus formation and to produce anthocyanins, and that inoculum size influenced cell growth in suspended cultures. In spite of these findings, the identification of anthocyanins in callus cultures from various parts of the strawberry has not been reported. Furthermore, various factors that influence cell growth and anthocyanin synthesis for consolidating the foundation of anthocyanin production using suspended cultures of strawberry cells remain to be elucidated.

Therefore, the objectives of this study are to establish a method to obtain friable calli and to determine the structures of major anthocyanins produced from suspended strawberry cells. In addition, the author examined various factors influencing cell growth and anthocyanin synthesis, such as auxin and cytokinin, cell inoculum size, sugar types, sucrose concentrations, total nitrogen, various ratios of ammonium and nitrogen, riboflavin and high sucrose concentrations and conditioning factors.

This research paper is divided into seven separate chapters according to procedures with the final chapter offering a conclusion of the study.

The contents of each chapter are described below.

Chapter 1. To produce secondary metabolites using plant cells, it is necessary to enhance cell growth. Therefore many plant tissues were dedifferentiated using various phytohormons. In obtaining friable calli from different parts of strawberry tissues, various phytohormonal conditions were investigated on solid culture medium, and the relationship between light intensity and phytohormons in the production of anthocyanins was further studied.

Chapter 2. After obtaining friable callus, it is very important to investigate what culture conditions are suitable for cell growth and production of secondary materials in suspension cultures. Therefore, the conditions of sub-cultures were studied for inducing anthocyanins in suspension cultures, and the anthocyanin structures were identified using the suspended callus. The effects of such factors as cell inoculum size, auxin and types of calli from different parts of the strawberry on cell growth and anthocyanin synthesis were also investigated in suspended cultures.

Chapter 3. The study of optimum culture medium was required to determine the maxium anthocyanin production in suspended cultures. Therefore, cell growth and anthocyanin synthesis were examined by applying various combinations of auxin and cytokinin, basic culture media, types of sugar, sucrose concentrations and various ratios of ammonium and nitrate.

Chapter 4. Riboflavin has been known as photoreceptor for induction of anthocyanin synthesis. Therefore, the possibility of increasing anthocyanin production through the application of riboflavin was studied in suspended cultures of strawberry cells.

Chapter 5. Since it was found that the conditioning factor promotes anthocyanin synthesis in suspended strawberry cultures of cells, the practical application of the conditioning factors to increase total anthocyanin production was investigated.

Chapter 6. Production costs and process design of anthocyanin production using suspended cultures of strawberry cells in commercial scale were studied.

Chapter 7. Conclusion.

CHAPTER 1

CALLUS FORMATION FROM THREE DIFFERENT PARTS OF STRAWBERRY TISSUES FOR ANTHOCYANIN PRODUCTION

INTRODUCTION

Plant tissue culturing is a technique which allows plant cells to grow on a solid or in a liquid medium, as is carried out with microorganisms. Reviewes by Shuler (1981), Curtin (1983) and Sahai and Knuth (1985) have revealed a tremendous potential for the development of plant cell culture systems to produce economically important compounds. Callus formation from strawberry fruits using benzyladenine (BA) and 2,4-dichlorophenoxy-acetic acid (2,4-D) was investigated by Asahira and Kano (1977). Although they found three types of calli were induced, they did not attempt to enhance anthocyanin formation. Hong *et al.* (1989a) also reported that three types of calli were formed even if cultivars differed, and they showed the influence of 2,4-D concentration on callus formation in the three different cultivars. However, calli formation derived from three different parts of a strawberry plant such as the apical meristem, leaf and petiole, remained elusive even with the addition of various kinds and concentrations of auxin, and combinations of BA and 2,4-D. Therefore, the objectives of this chapter are to establish a method of tissue culture to obtain friable calli from different parts of strawberry and to examine the influence of auxin and cytokinin on callus formation and anthocyanin accumulation.

MATERIALS AND METHODS

Plant Materials

Callus tissues were induced from the apical meristem, leaf, and petiole of *Fragaria ananassa* cv Shikinari. The excised runners (30~50 mm in length) were obtained from a

greenhouse-grown strawberry (Fig. 1). They were sonically cleaned in 70% ethanol and sterilized with a 5% sodium hypochlorite solution, and then rinsed with sterile water three times. The apical meristems (0.2 mm in length) were excised under a dissecting microscope from the runners. Leaves and petiols were obtained from aseptically regenerated plants which were induced on the LS solid medium supplemented with 5 mg/L of BA cultured for one month under 3000 lux.

Auxin on callus formation

Apical meristem, leaf and petiole were placed on the LS (Linsmaier and Skoog, 1965) medium containing 3% sucrose, 0.2% Gellangum (Wako Chemical) and various kinds of auxin. The used auxins were 2,4-D, 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). These concentrations were changed from 0.1 mg/L to 5 mg/L. Tissues were incubated for two monthes at 25 ℃ under a 16 h-light, 8 h-dark cycle with light intensity of 800 lux. Callus tissues were transferred every 3 weeks to a freshly prepared LS medium.

Combinations of 2,4-D and BA

To obtain more suitable phyto-hormonal conditions, combinations of BA and 2,4-D were examined. Tissues were incubated at 25 ℃ for 2 months under a 16 h-light, 8 h-dark cycle with light intensity of 800 lux. Callus tissues were transferred every 3 weeks to a freshly prepared LS medium. After obtaining friable calli, they were transferred to freshly prepared LS solid medium and then placed under light at 8000 lux for 3 weeks. Anthocyanin accumulation was observed after this.

RESULTS AND DISCUSSION

Auxin on callus formation

Kinds of growth regulators and their concentrations in the culture medium are critical to control callus growth and morphogenesis, as first indicated by Skoog and Miller (1957).

Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promote cell proliferation with the formation of callus. Often, 2,4-D is used only to initiate callus.

Various types of calli were induced with auxins, however, white friable callus (Fig. 2) formation from strawberry tissues was observed only in a media containing 2,4-D as indicated (Table 1). The 2,4-D concentration was above 0.5 mg/L, and there was almost no difference among apical meristems, leaf and petiol. On the other hand, the other three auxins produced brown calli (Fig. 3), and no white friable callus were formed on these three auxins. In the concentration below 1 mg/L of these three auxins, no callus formation was observed. Considering the effects of auxin for callus formation, 2,4-D was best for obtaining white friable callus.

Combinations of 2,4-D and BA

Effects of different combinations of 2,4-D and BA on the initiation of callus from the apical meristems, leaf tissue and petiole are shown in Table 2. After two months of culture, white friable calli were observed on the agar medium, but their formation was observed only when the concentration of 2,4-D was 0.5 mg/L or higher and that of BA lower than 2 mg/L. These white friable calli were inducible when incubated below 800 lux. When placed under 3000 lux of light, the tissues formed green or compact-surfaced calli (Fig. 4). Especially, strawberry plant was regenerated when tissues were cultured on the media supplemented BA above 1 mg/L under light at 3000 lux (Fig. 5). No essential differences in initiation of white friable calli were observed among leaf, apical meristem, and petiole tissues.

Asahira and Kano (1977) and Hong *et al.* (1989a) reported the induction of white friable callus from strawberry fruits. The former authors obtained three types of calli (white friable, green firm, and pale green firm calli). The white calli were obtained in MS medium with either 0 or 0.1 mg/L of BA and either 1 or 5 mg/L of 2,4-D. Hong *et al.* (1989a) also reported that the ability to initiate white friable callus was different among cultivars. They obtained the callus from "Aptos" and "Brighton" on MS medium

containing various concentrations of 0.5, 1.0, 5.0, and 10 mg/L of 2,4-D. These data suggests that there is a range in both 2,4-D and BA to initiate white friable callus, and that it is important to culture tissues at 800 lux. While, by supplementing BA in the media, white friable callus seemed to form.

Combinations of 2,4-D and BA under 3000 lux

In the present study, red pigments began to accumulate on the surface of each callus two weeks after transferring light intensity irradiating on the white friable callus from 800 lux to 3000 lux (Fig. 6). The intensity of the red color continued to increase until week two, after that the pigment turned dark brown. On the other hand, calli in the dark remained white throughout the entire experiment. In particular, a combination of 2,4-D of 1 mg/L and BA of 0.1 mg/L induced the best pigmentation (Table 3). Hong *et al.* (1989a) obtained similar results, without testing the matrix of the auxin and cytokinin for pigment accumulation. The effect of light on formation of pigment has also been noted in carrot cells by Dougall *et al.* (1980). Therefore, the results clearly suggested that light significantly enhanced red pigment formation in strawberry calli. While, cells which have high anthocyanin productivity have been selected by continuous clonal selection (Yamamoto *et al.*, 1982; Sakamoto *et al.*, 1993).

However, clonal selection of callus accumulating red pigment has not been successful in strawberry "Shikinari" cell. After accumulation of anthocyanin on the surface of the callus, color of the cells turned brown and cells lost potentiality to proliferate. Hong *et al.* (1989a) also did not select the cells of high anthocyanin production. This may indicate clonal selection of callus producing high anthocyanin content is difficult in a strawberry cell.

CONCLUSION

1. 2,4-D was the best auxin to enhance callus formation from the tissues of three different parts of the strawberry plant (i.e. leaf, apical meristem and petiole), and the

concentrations above 0.5 mg/L were necessary to induce white friable calli only under dim lighting (800 lux).

2. The white friable calli from the three different parts were obtained only when the concentration of 2,4-D was 0.5 mg/L or higher and that of BA lower than 2 mg/L, and the tissues were cultured under a dim light (800 lux).

3. Anthocyanin accumulation on the surface of calli was observed by applying the approximate combination of 1 mg/L 2,4-D and 0.1 mg/L of BA under 3000 lux.

Table 1 Effects of various auxin on calli formation from apical meristem, leaf and petiole tissues

- O White friable callus was induced.
- Δ Browning callus was induced.
- × No callus was induced.

apical meristem (mg/L)	0.1	0.5	1.0	2.0	5.0
2,4-D	×	O	O	O	O
NAA	×	×	Δ	Δ	Δ
IBA	×	×	×	Δ	Δ
IAA	×	×	Δ	Δ	Δ

Leaf (mg/L)	0.1	0.5	1.0	2.0	5.0
2,4-D	×	O	O	O	O
NAA	×	×	Δ	Δ	Δ
IBA	×	×	Δ	Δ	Δ
IAA	×	×	Δ	Δ	Δ

petiole (mg/L)	0.1	0.5	1.0	2.0	5.0
2,4-D	×	O	O	O	O
NAA	×	×	×	Δ	Δ
IBA	×	×	Δ	Δ	Δ
IAA	×	×	Δ	Δ	Δ

Table 2A Influence of 2,4-D and BA on the callus formation of the strawberry apical meristem tissue cultured under 800 lux

BA (mg / L) \ 2,4-D (mg / L)	0	0.1	0.5	1	2	5
0	0/5	0/5	4/5	4/5	4/5	4/5
0.1	0/5	0/5	4/5	5/5	5/5	5/5
0.5	0/5	0/5	4/5	5/5	5/5	5/5
1.0	0/5	0/5	4/5	4/5	5/5	2/5
2.0	0/5	0/5	5/5	4/5	4/5	1/5
5.0	0/5	0/5	1/5	0/5	1/5	0/5

Each value represents the number of white friable calli formed out of five explants.

Table 2B Influence of 2,4-D and BA on the callus formation of the strawberry leaf tissue cultured under 800 lux

BA (mg / L) \ 2,4-D (mg / L)	0	0.1	0.5	1	2	5
0	0/5	0/5	4/5	4/5	4/5	5/5
0.1	0/5	0/5	5/5	5/5	5/5	5/5
0.5	0/5	0/5	5/5	5/5	5/5	4/5
1.0	0/5	0/5	4/5	4/5	5/5	2/5
2.0	0/5	0/5	5/5	4/5	4/5	1/5
5.0	0/5	0/5	1/5	0/5	1/5	0/5

Each value represents the number of white friable calli formed out of five explants.

Table 2C Influence of 2,4-D and BA on the callus formation of the strawberry petiole tissue cultured under 800 lux

BA (mg / L) \ 2,4-D (mg / L)	0	0.1	0.5	1	2	5
0	0/5	0/5	4/5	4/5	4/5	4/5
0.1	0/5	0/5	5/5	5/5	5/5	5/5
0.5	0/5	0/5	5/5	5/5	5/5	1/5
1.0	0/5	0/5	2/5	4/5	5/5	2/5
2.0	0/5	0/5	1/5	4/5	4/5	1/5
5.0	0/5	0/5	0/5	0/5	1/5	0/5

Each value represents the number of white friable calli formed out of five explants.

Table 3A Influence of light irradiation on anthocyanin accumulation on the surface of white friable calli derived from the strawberry apical meristem tissue under a light of 8000 lux

BA (mg / L) \ 2,4-D (mg / L)	0.5	1	2	5
0	1/5	2/5	2/5	0/5
0.1	1/5	2/5	1/5	2/5
0.5	2/5	2/5	3/5	2/5
1.0	2/5	3/5	3/5	1/5
2.0	1/5	2/5	2/5	1/5
5.0	0/5	0/5	1/5	0/5

Each value represents the number of red pigmented calli formed out of five explants.

Table 3B Influence of light irradiation on anthocyanin accumulation on the surface of white friable calli derived from the strawberry leaf tissue under a light of 8000 lux

BA (mg / L)	2,4-D (mg / L) 0.5	1	2	5
0	1/5	2/5	2/5	2/5
0.1	2/5	5/5	1/5	2/5
0.5	2/5	4/5	3/5	2/5
1.0	2/5	3/5	3/5	1/5
2.0	1/5	2/5	2/5	1/5
5.0	1/5	0/5	1/5	0/5

Each value represents the number of red pigmented calli formed out of five explants.

Table 3C Influence of light irradiation on anthocyanin accumulation on the surface of white friable calli derived from the strawberry petiole tissue under a light of 8000 lux

BA (mg / L)	2,4-D (mg / L) 0.5	1	2	5
0	0/5	2/5	2/5	0/5
0.1	0/5	5/5	1/5	2/5
0.5	0/5	4/5	3/5	2/5
1.0	2/5	3/5	3/5	1/5
2.0	1/5	2/5	2/5	1/5
5.0	0/5	0/5	1/5	0/5

Each value represents the number of red pigmented calli formed out of five explants.

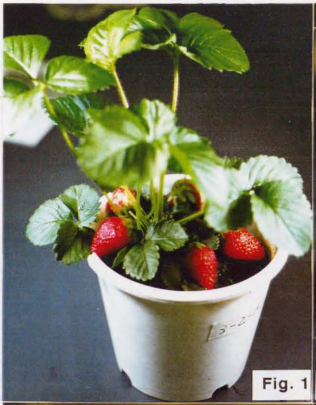


Fig. 1

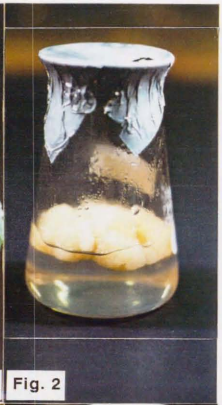


Fig. 2

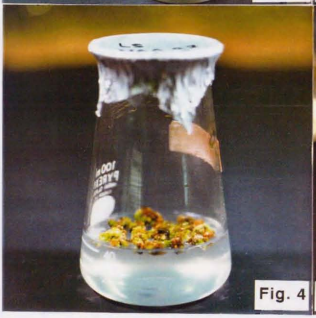


Fig. 4

Fig. 3

Fig. 1 *Fragaria ananassa* cv Shikinari grown in greenhouse.

Fig. 2 White friable callus cultured on LS medium containing 2,4-D (1 mg/L), sucrose 3% (W/V) and 0.2% Gellangum under 800 lux.

Fig. 3 Brown callus produced on LS medium containing NAA (1 mg/L), sucrose 3% (W/V) and 0.2% Gellangum under 800 lux.

Fig. 4 Green or compact-surfaced calli produced on LS medium containing 2,4-D (1mg/L), BA (0.1 mg/L) sucrose 3% (W/V) and 0.2% Gellangum under 3000 lux.

Fig. 5

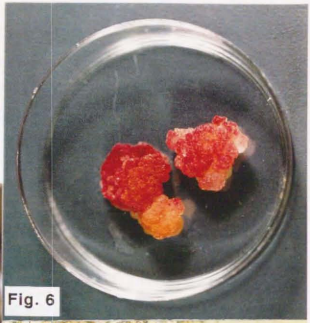


Fig. 6

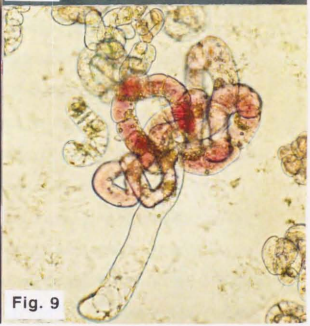


Fig. 9

Fig. 5 Regenerated strawberry plant when tissue was cultured on LS medium containing BA (1 mg/L) sucrose 3% (W/V) and 0.2% Gellangum under 3000 lux.

Fig. 6 Pigments accumulation on the surface of white friable calli under 3000 lux.

Fig. 9 Anthocyanins accumulation in vacuoles of strawberry suspension cells cultured under 8000 lux.

CHAPTER 2

ANTHOCYANIN PRODUCTION AND IDENTIFICATION OF MAJOR PIGMENTS USING SUSPENSION CULTURE OF STRAWBERRY CELLS

INTRODUCTION

The main anthocyanin pigment in intact strawberries was identified as pelargonidin-3-glucoside (Robinson and Robinson, 1932). Lukton *et al.* (1955) identified a second minor pigment in cultivated strawberries as cyanidin-3-glucoside. Furthermore, Henry and Markakis (1968) and Wrolstad *et al.* (1970) identified additional minor pigments as quercetin-3-glucoside, kaempferal-3-glucoside and leucocyanidins. Although, Hong *et al.* (1989a) reported that cultivars were different in their ability to initiate callus formation and to produce anthocyanin, and that the inoculum size influenced cell growth in suspension cultures, the identification and production of anthocyanin in callus cultures from various parts of strawberry plants have not been reported.

Therefore, the objectives of this chapter are to show the method of sub-suspended cultures of white friable callus to produce anthocyanin in suspension. In addition, the author will demonstrate the identification of pigments through the use of FAB-MS and NMR, demonstrate cell growth and anthocyanin synthesis in suspended cultures from different parts of the strawberry plant, and discuss the influence of auxins and cell inoculum size on anthocyanin production.

MATERIALS AND METHODS

Sub-suspension Cultures of Friable Callus

Cell suspension cultures were initiated by transferring about 2 g (fresh weight) of the friable callus derived from apical meristem grown on the medium supplemented with 2,4-D (1 mg/L) and BA (0.1 mg/L) to 100 mL of liquid LS medium which was supplemented

with 3% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 ml flasks. After pH was adjusted to 5.7 using HCl and KOH, the medium was autoclaved at 110 ℃ for 20 min. Cells were incubated in a rotary shaker (80 rpm) under 800 lux at 25 ℃. The suspension cultures were grown for 5 weeks, while the medium was changed every week. The inoculation rate was about 10%. The resulting cell suspension of each week (2 g-fresh weight) was transferred to the fresh liquid LS medium and then incubated under a light intensity of 8000 lux for 2 weeks at 25 ℃ on a rotary shaker (80 rpm) for examining best sub-culture duration of anthocyanin production by measuring anthocyanin content.

Callus from Different Parts and Inoculum Size

Suspended cell cultures from different parts of strawberry tissues (apical meristems, leaf and petiol) were initiated by transferring about 2 g (fresh weight) of the each friable callus tissue grown on 2,4-D (1 mg/L) and BA (0.1 mg/L) to 100 mL of liquid LS medium supplemented with 3% sucrose and 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks, and by incubating on a rotary shaker (80 rpm) under 800 lux at 25 ℃. The suspension cultures were grown for 3 weeks, meanwhile the medium was changed every week. The inoculation rate was about 10%. The resulting cell suspension (2 g fresh) was transferred to the fresh liquid LS medium and then incubated under a light intensity of 8000 lux at 25 ℃ on a rotary shaker (80 rpm) for anthocyanin production. After 2 weeks of incubation, cells were harvested and the cell weight and anthocyanin content were measured. Inoculation size (initial concentration at 1 to 10 g fresh cell weight /100 mL) was also changed and the cells were cultured for 20 days using callus from apical meristem under 8000 lux.

Effects of Auxins

To examine the influence of auxins on cell growth and anthocyanin contents, cells derived from apical meristems and precultured for 3 weeks with 2,4-D (1 mg/L) and BA (0.1 mg/L) were cultured in the liquid LS medium containing NAA, IAA, IBA, or 2,4-D for 2 weeks under 8000 lux.

HPLC Analysis

For HPLC analysis, fresh callus tissues from the leaf, petiole and apical meristem were extracted for anthocyanins using the 35% solution A (acetic acid : acetonitrile :water-20:25:55) containing 0.1% trifluoroacetic acid (TFA) at 4 ℃. After filtration, analytical HPLC (Waters 600E) was carried out using octadecyl silica (ODS) column (Develosil ODS-5 4.6 mm ϕ X 250 mm, Nomura Chemical) eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40 ℃. Anthocyanins were analyzed using a pair of JASCO 880-PU pumps equipped with a MULTI-340 photo-diode array detector. HPLC using an Asahipak-ODP column (4.6 X 250 mm) was used and eluted with a linear gradient elution from 10% to 30% aq.acetonitrile containing 0.5% TFA in 30 min at 40 ℃.

Extraction and Isolation of Anthocyanin

Fresh cells (500 g) were extracted overnight for anthocyanin using a solution of 0.1% TFA-methanol (MeOH) at 4 ℃. Cells were filtered, then the filtrate was condensed to about 20% of volume and poured into an Amberlite XAD-7 column (50 x 250 mm). Pigments were eluted stepwise from H_2O to 50% aq CH_3CN containing 1% TFA. They were concentrated in the 30 % fraction of CH_3CN. The solution was evaporated below 35 ℃, and the concentrate was purified using a preparative ODS (glass column, 20 x 1000 mm) and a methanol solution (water:acetic acid :methanol-80:15:5). Further purification was carried out using a preparative ODS stainless-steel column (20 x 250 mm, Develosil ODS-5, Nomura Chemical) with solution A diluted to 30% and containing 0.1% TFA. After freeze-drying, the pigments were used for structural identification of anthocyanin.

Acid Hydrolysis of Anthocyanins

The anthocyanidin nucleus was identified with acid hydrolysis of anthocyanins followed by HPLC analysis comparing with authentic samples. Anthocyanin (1 mg) was dissolved in 0.5 mL of 6 N HCl - MeOH (1:1) and heated at 80 ℃ for 6 hr. The

reaction mixture was condensed *in vacuo*, filtered, and then analyzed by HPLC. The sample solution was co-injected into the HPLC column with authentic cyanidin produced by acid hydrolysis from commercially available cyanin and authentic peonidin obtained from acid hydrolysis of peonin isolated from red sepals of *Fuccia hybrida*, respectively.

Spectroscopic Analysis

Purified anthocyanin from the leaf callus was dissolved in 0.01% HCl-MeOH, and its UV and visible spectra were recorded on a Hitachi UV-228 spectrometer. The ^{1}H-NMR (500 MHz) spectra were obtained on a JEOL GX-500 spectrometer in a 5 mm ϕ tube at variable temperatures using a 10% TFA*d*-CD_3OD solution as a solvent. Chemical shifts were recorded in parts per million downfield from internal tetramethylsilane (TMS) as a standard. The FAB-mass spectrum was recorded using JEOL DX304/DA5000 systems and a 1N HCl-glycerol matrix.

Determination of Anthocyanin and Cell Growth

Fresh callus tissues were extracted overnight using a solution containing 0.1% HCl-MeOH at 4 ℃. After centrifugation at 1000 x g for 5 min, the absorbance of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated with the extinction coefficient ($E^{1\%}_{1cm}$=680 at 528 nm) which was obtained by using purified peonidin-3-glucoside from cultured strawberry cells as a standared. Total anthocyanin yield was expressed as mg/100 mL of liquid LS medium in a 500 mL cell culture flask. Cells were separated from the culture medium by filtration through a nylon filter (30 μm) and then weighed. Results were expressed as fresh cell weight per flask.

Statistical analysis

Data was presented as means ± SE (Standard error). Statistical analyses were made using one-way analysis of variance (ANOVA). Differences with P (Probability) < 0.01 were considered statistically significant.

RESULTS AND DISCUSSION

Sub-suspension Cultures of Friable Callus

Usually plant callus cells produce only small amounts of secondary metabolites. Except a few examples, such as vitamin B6 (Yamada and Watanabe, 1980), alkaloid (Zenk *et al.*, 1977; Ogino *et al.*, 1978) and ubiquinone (Matsumoto *et al.*, 1980), callus strains contain amounts of secondary metabolites less than those found in intact plants. Anthocyanin was also contained a little in cultured cells in the same manner as mentioned above. Especially, only a small amount of anthocyanins is produced on the surface of the callus cultured on solid medium. Therefore, it is necessary to develop culture system for suspension culture. The result of this experiment showed that only a little anthocyanin was produced when a callus grown on solid medium was directly transferred to the LS liquid medium and cultured at 8000 lux (Fig. 7). However after three times of sub-culture (1-week intervals), suspended callus was capable to produce anthocyanin. There were no significant differences between average anthocyanin synthesis at 3 week and 5 week culture duration, therefore, at least three repetitions of sub-culture (1-week intervals) under 800 lux was needed to produce anthocyanin at 8000 lux stably.

Callus from Different Parts and Inoculum Size

Time course and suitable inoculum sizes for anthocyanin production in suspended cultures derived from apical meristem (Fig. 8) indicated inoculum sizes influenced cell weight and anthocyanin production. Cell growth was remarkably stalled at inoculation levels less than 2 g/100 mL. The higher inoculum levels induced rapid cell growth during the first two weeks and reached each stationary phase at about 15 days. These cells began to lyse by the end of the third week. This difference may be due to factors such as cell competition and/or interaction with limiting nutrient supply for cell growth. It is important for anthocyanin production to preculture calli for at least 3 weeks under 800 lux. Bright red pigments were first observed in vacuoles of cells after 2 days in

culture (Fig. 9) and they then accumulated in the cells. Anthocyanin content was remarkably influenced by inoculation size. The highest content (88 μg/g fresh cell) was obtained with 2 g inoculum (Fig. 8b). In higher inoculum sizes of 5 g and 10 g cells, anthocyanin contents reached stationary low levels at 4 days. Anthocyanin content peaked at 2 weeks in an inoculation size of 1 g cells but its level was low. During the time course of period when the anthocyanin content was monitored, a shoulder occasionally occurred (Fig. 8b). We cannot explain the reason for this. Considering total anthocyanin production per flask (Fig.8c), inoculum size of about 2 g cells was best for anthocyanin production in a 500 mL flask containing 100 mL medium. Total anthocyanin reached the maximum of 3 mg/flask at 2 weeks. Inoculum sizes of 5 g and 10 g cells produced only about half the total anthocyanin compared with the 2 g inoculum. Hong *et al.* (1989a) reported the growth curve of cells in liquid suspension culture of strawberry fruits. That curve was almost the same as the cell growth-inoculum size relationship curve of our present study. However, the relationship between inoculum size and anthocyanin production was not established in their study.

Cell growth and anthocyanin production were compared among apical meristem, leaf and petiole tissues (Fig. 10). The petiole was considerably less effective than the other tissue parts in terms of both cell growth and pigment formation (65.6 μg/g callus). Cell weight and anthocyanin content were almost the same between the apical meristem (88.2 μg/g callus) and the leaf callus (108.7 μg/g callus) as shown in Fig. 10b. However, the total anthocyanin was significantly ($P < 0.01$) higher in the leaf than the apical meristem (Fig. 10c). Hong *et al.* (1989a) produced anthocyanin from immature strawberry fruits and reported the absorbance of anthocyanin content as 0.45 /g callus at 520 nm. This led to an anthocyanin content of about 66 μg/g callus. On the other hand, Pilando *et al.* (1985) reported anthocyanin content of intact strawberry fruits (Benton and Totem varieties). They showed the total anthocyanin content reached about 32.7 mg and 50.3 mg/100 g-fresh weight in fully ripe strawberries of Benton and Totem var, respectively. These results indicated that, although anthocyanin was producible in culture from various parts of strawberry, there were wide differences in ability for anthocyanin

production between parts of strawberry. Production ability ranked as leaf, apical meristem, immature fruit, and petiole in descending order, and the anthocyanin content from leaf callus almost reached one-third of the intact Benton strawberry fruits.

Effects of Auxins

Of the four different auxins used, 2,4-D was most effective for anthocyanin production with the callus derived from apical meristem (Fig. 11c). It consistently stimulated cells to produce anthocyanin during 2 weeks. IAA was highly effective for anthocyanin production only during the first week (data not shown). Thereafter, anthocyanin production decreased, probably due to decomposition of IAA by light or other factors. Since only 2,4-D was used in this experiment (Fig. 11), the total production of anthocyanin was low compared with the apical meristem in which 2,4-D was used together with BA (Fig. 8).

Analysis of Anthocyanin

Eight kinds of anthocyanins were detected with HPLC at 528 nm in all cultured callus tissues used. Two variations were isolated (Fig. 12) by using an ODS column (Yoshida *et al.*, 1990). Peak 1 anthocyanin (**1**) and peak 2 anthocyanin (**2**) comprised almost 15% and 70% of the total anthocyanin, respectively. No differences in anthocyanin composition and the number of major peaks were found among these calli, indicating that the control of the enzymatic function for anthocyanin production in cells was almost the same by dedifferentiation. Electronic absorption spectra (λ_{max} 520 nm in 0.01N HCl-MeOH) of the pigments suggest that the nuclei of chromophores were cyanidin and/or peonidin. The structures of aglycone of **1** and **2** were determined by acid hydrolysis followed by HPLC and UV spectral analysis (Fig. 13). By acid hydrolysis **1** gave cyanidin and **2** gave peonidin. FABMS of **1** gave a molecular ion peak (M^+) at *m/z* 449 (Fig. 14). The ^{1}H-NMR spectrum of **1** (Fig. 15 and Table 4) showed the presence of one cyanidin nucleus and one hexose. On the basis of H-4 (9.01, S), all of the signals at the aromatic moiety could be assigned by a homo spin decoupling

experiment; the vicinal coupling of the sugar signals was $J_{1,2}$≅7.5Hz, $J_{2,3}$≅$J_{3,4}$≅$J_{4,5}$≅9.5Hz, indicating that the sugar was a β-D-glucopyranoside. By irradiation of the anomeric proton we observed +10% NOE at H-4, indicating that the linkage position of the sugar was OH at the 3-position of the nucleus. Consequently, **1** must be cyanidin-3-glucoside (Fig. 16).

A molecular ion peak of **2** appeared at *m/z* 463 (Fig. 17). The NMR spectrum (Fig. 18 and Table 5) was very similar to that of **1** except for the presence of one CH_3O-. By irradiation of CH_3O and the anomeric proton we observed +15% NOE at H-2' and +10% at H-4, respectively, indicating that the anthocyanidin was peonidin and the sugar was attached to OH at 4-position of the nucleus. These results confirmed that **2** is peonidin-3-glucoside (Fig. 19). Each electronic spectral data are shown in Fig. 20 and 21.

There have been many studies on identification of anthocyanin pigments in intact strawberries. The main pigment has been identified as pelargonidin-3-glucoside (88%) and the second major pigment as cyanidin-3-glucoside (12%) (Wrolstad and Putnam, 1969). Hong and Wrolstad (1990) also reported relative percentages of cyanidin (24%) and pelargonidin (75%) based on total peak area from HPLC. However, the main anthocyanin produced in the present tissue cultures was a peonidin-derivative.

Although both pelargonidin- and cyanidin-type anthocyanins have been reported as major pigments in intact strawberry fruits, it is interesting that the callus tissues of strawberries were capable of producing peonidin- and cyanidin-type anthocyanins. Yamakawa *et al.* (1983a) reported a difference in anthocyanin composition between cultured cells and intact organs in *Vitis* sp. They found that the intact pericarp contained malvidin- and peonidin-glucoside, whereas callus tissues from grape vines produced cyanidin- and peonidin-glucoside. Mizukami *et al.* (1988) also reported the difference in anthocyanin compositions between cultured cells and intact organs. Those reports suggested that the shikimic acid channel for producing anthocyanin was inhibited by an unknown factor during callus formation. As a result, peonidin- and cyanidin-type anthocyanins may be produced in the growth of callus tissues.

CONCLUSION

1. To produce anthocyanins in suspended cultures using callus cultured on solid medium, cells must be sub-cultured at least three sequential times (1-week intervals) under dim lighting (800 lux).

2. Cell inoculum size influenced cell growth and anthocyanin synthesis. The highest anthocyanin production was obtained using 2 g of fresh cell inoculum after culturing for two weeks.

3. 2,4-D was also the most efficient auxin for anthocyanin production in suspended cultures of strawberry cells.

4. Cell growth and anthocyanin synthesis using leaf callus were higher than that obtained from other tissues, and productivity ranked as leaf, apical meristem and petiole in descending order.

5. About eight kinds of anthocyanins were detected with HPLC at 528 nm in all cultured callus tissues and, using FAB-MS and NMR, the two major anthocyanins were identified as cyanidin-3-glucoside and peonidin-3-glucoside.

Table 4 Assignment of the ^{1}H-NMR Spectra of Peak 1 anthocyanin (10% TFAd-CD_3OD at 20 °C, 500MHz)

9.04(1H, S, H-4), 8.27(1H, dd, J=9.5&2.5Hz, H-6′), 8.07 (1H, d, J=2.5Hz, H-2′), 7.04 (1H, d, J=9.5 Hz, H-5′), 6.91 (1H, dd, J=2.5 & 0.5Hz, H-8), 6.68 (1H, d, J=2.5Hz, H-6), 5.33 (1H, d, J=7.5Hz, G-1), 3.70 (1H, dd, J=9.5 & 7.5Hz, G-2), 3.57 (1H, t, J=9.5Hz, G-3), 3.48 (1H, t, J=9.5Hz, G-4), 3.58 (1H, ddd, J=9.5, 6.5 & 2.5Hz, G-5), 3.73 (1H, dd, J=12.5 & 6.5Hz, G-6a), 3.91 (1H, dd, J=12.5 & 2.5Hz G-6b), NOE G-1→H-4 (9% enhancement)

Table 5 Assignment of the ^{1}H-NMR Spectra of Peak 2 anthocyanin (10% TFAd-CD_3OD at 20 °C, 500MHz)

9.08 (1H, S, H-4), 8.28 (1H, dd, J = 9Hz & 2.5Hz, H-6′), 8.26 (1H, d, J = 2.5Hz H-2′), 7.09 (1H, d, J = 9.0Hz, H-5′), 8.96 (1H, d′, J = 2.5 & 0.5Hz, H-8), 6.00 (1H, b, d, J = 2.5Hz, H-6), 5.34 (1H, d, J = 7.5Hz, G-1), 4.04 (3H, S, OCH3), 3.94 (1H, dd, J = 12.5 & 2.5Hz, G-6a), 3.71 (1H, dd, J = 12.5 & 6Hz, G-6b), 3.98 (1H, dd, J = 7.5 & 9.0Hz, G-2), 3.59 (1H, ddd, J = 12.5, 6, 2.5Hz, G-5), 3.57 (1H, t. J = 9.0Hz, G-3), 3.45 (1H, t, J = 9.0, G-4), NOE OCH3→H-2′ (+13%), G-1-H-4 (+ 10%),

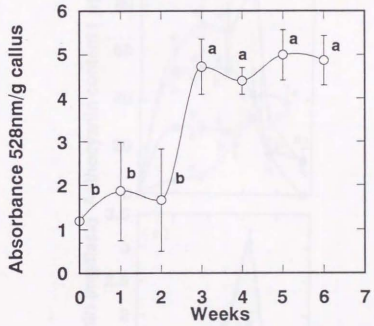


Fig. 7 The effect of subculture durations under 800 lux for anthocyanin production. Cells (2g fresh) were cultured in LS medium containing 2,4-D (1 mg/L), BA (0.1 mg/L) and sucrose 3% (W/V) under 8000 lux for 2 weeks after various durations of subculture under 800 lux. The symbols a-b mean that there are no significant differences at P= 0.01 in the ordinate values of the same symbols.

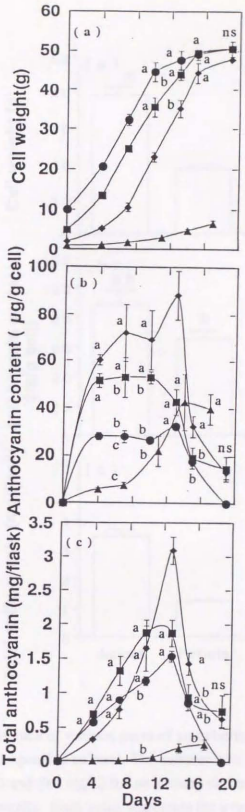


Fig. 8 Influence of inoculum sizes on cell growth and anthocyanin production in suspension cultures of strawberry. The cultures were incubated on a rotary shaker (80 rpm) under continuous light (8000 lux). Initial fresh cell weight, ●: 10g/100mL; ■ :5g/100mL; ◆ : 2g/100mL; ▲ :1g/100mL. Each value represents the average of three replicates and vertical lines represent the standard error of replicates.

The symbols a-c mean that there are no significant differences at P= 0.01 in the ordinate values of the same symbols. ns: Not significant within the same day.

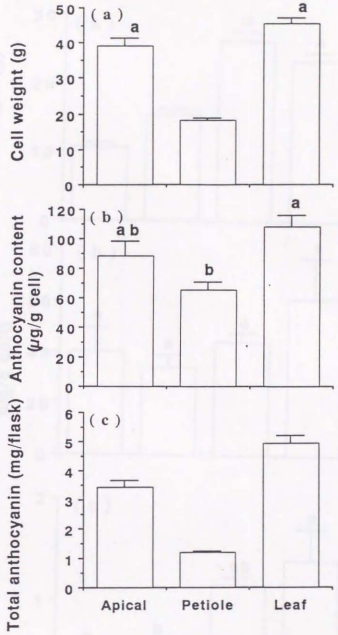


Fig. 10 Influence of various parts of strawberry on cell growth and anthocyanin production in suspension cultures. The cultures were maintained in an LS medium with (1 mg/L) 2,4-D and (0.1 mg/L) BA on a rotary shaker (80 rpm) under light (8000 lux) at 25°C for two weeks. Each value represents the average of three replicates and vertical lines represent the standard error of replicates. a-b: Bars with same letter do not show significant difference at P= 0.01.

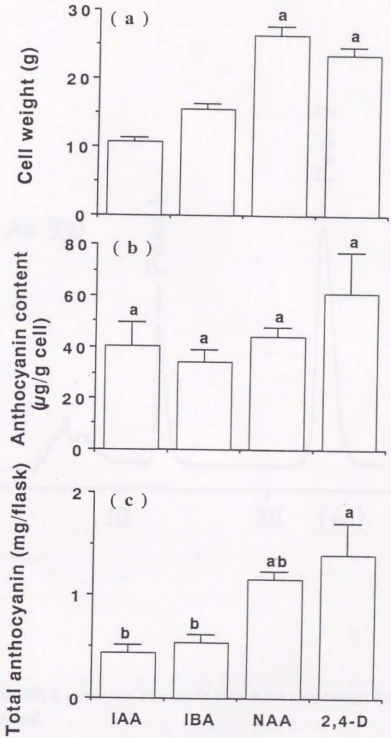


Fig. 11 Effects of auxins on cell growth and anthocyanin production in suspension cultures of strawberry cells. The cultures were maintained in an LS medium with auxin (1 mg/L) on rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for two weeks. Vertical lines represent standard errors for three flasks. a-c: Bars with same letter do not show significant difference at P= 0.01.

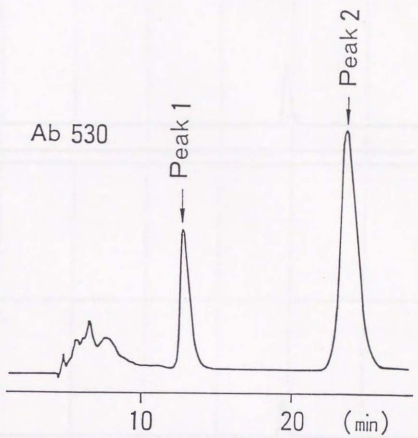


Fig. 12 The HPLC separation of a crude 35% A solution extract from callus tissue derived from leaf.

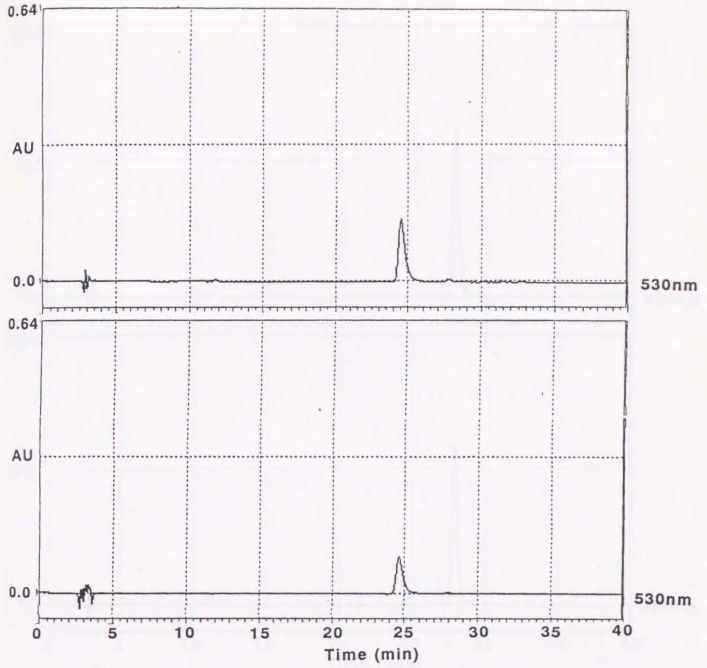


Fig. 13A Chromatograms of the authentic cyaninidin (up side) and anthocyanidin-1 after acid hydrolysis of peak 1 (down). Analytical HPLC was carried out using octadecyl silica (ODS) column eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40°C.

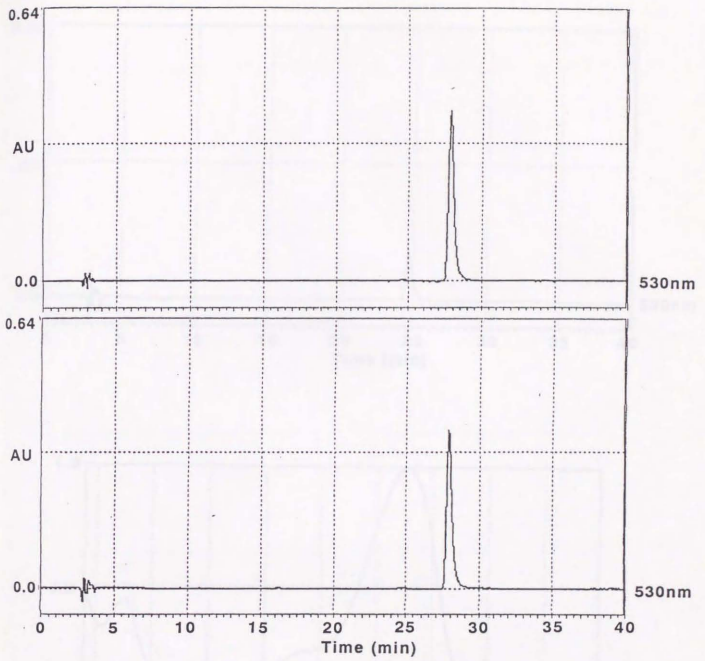
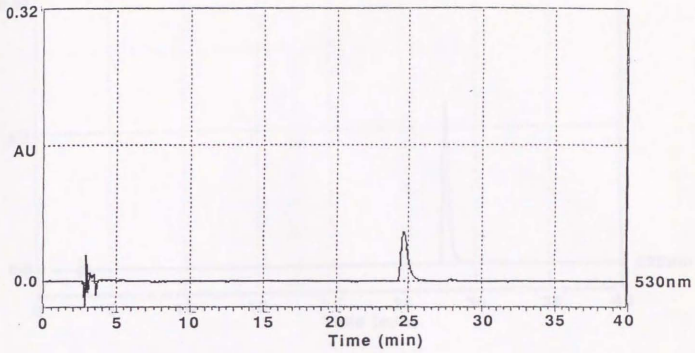


Fig. 13B Chromatograms of the authentic peonidin (up side) and anthocyanidin-2 after acid hydrolysis of peak 1 (down). Analytical HPLC was carried out using octadecyl silica (ODS) column eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40°C.



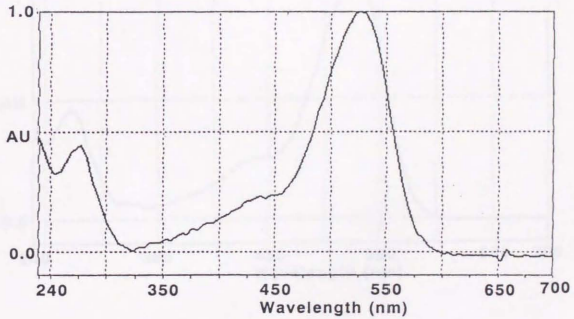


Fig. 13C Chromatograms of the co-injected authentic cyaninidin and anthocyanidin-1 which was acid hydrolyzed of peak 1 (up side) and the UV spectra. Analytical HPLC was carried out using octadecyl silica (ODS) column eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40°C.

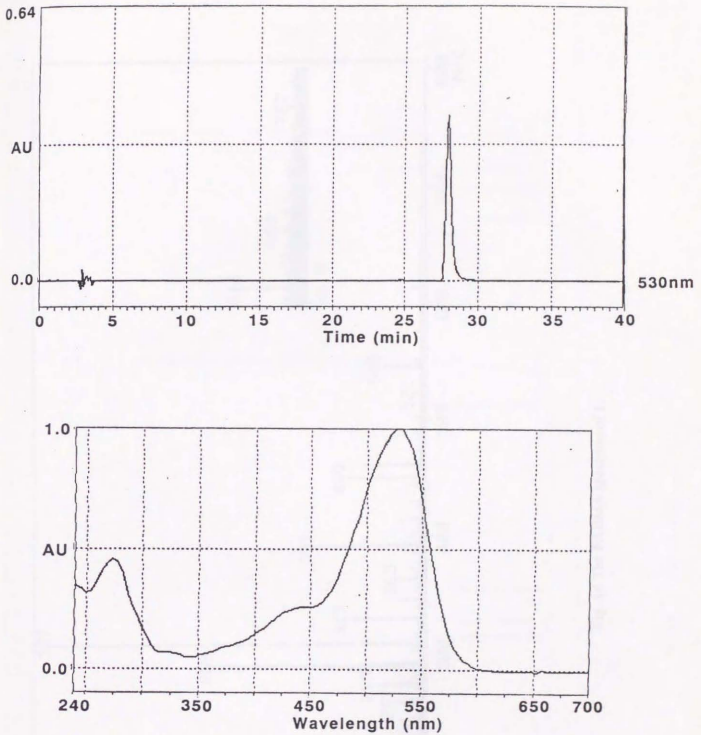


Fig. 13D Chromatograms of the co-injected authentic peoninidin and anthocyanidin-2 which was acid hydrolyzed of peak 2 (up side) and the UV spectra. Analytical HPLC was carried out using octadecyl silica (ODS) column eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40°C.

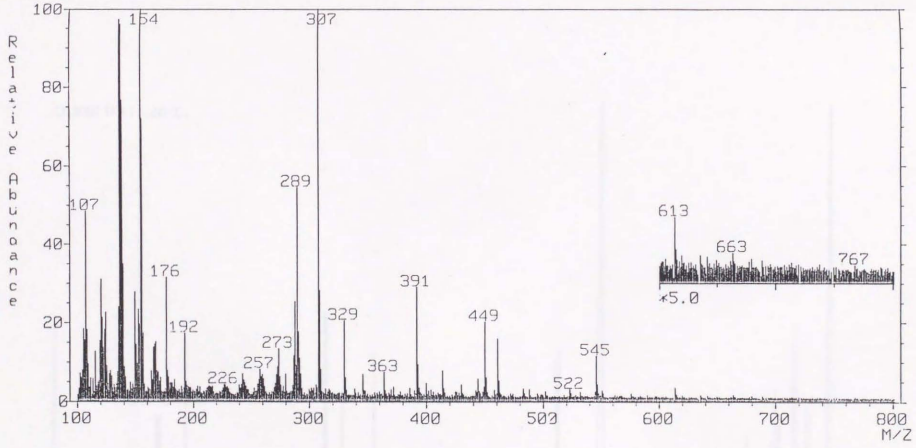


Fig. 14 The FABMA spectrum of 1.

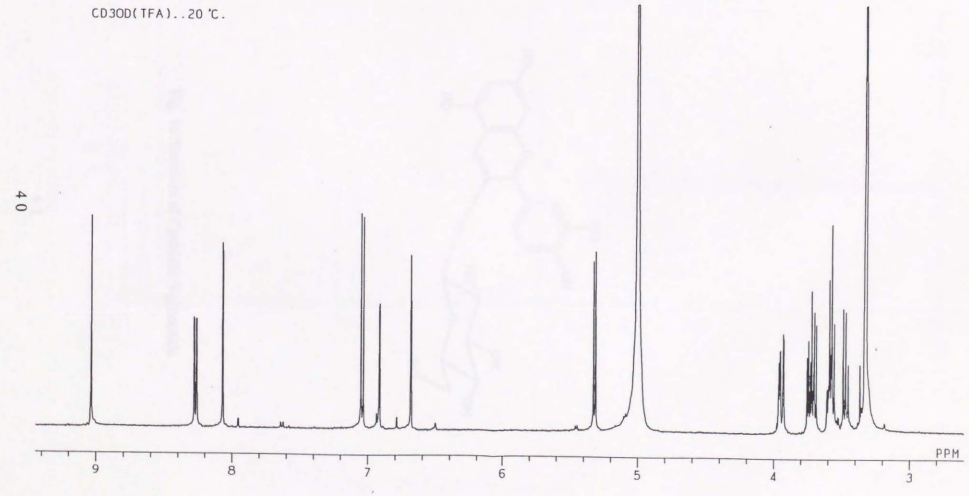


Fig. 15 The 1H-NMR spectrum of 1.

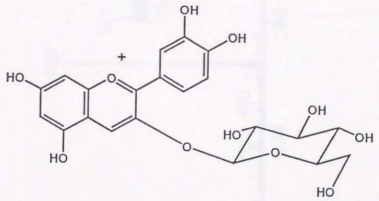


Fig. 16 Structure of Cyanidin-3-glucoside.

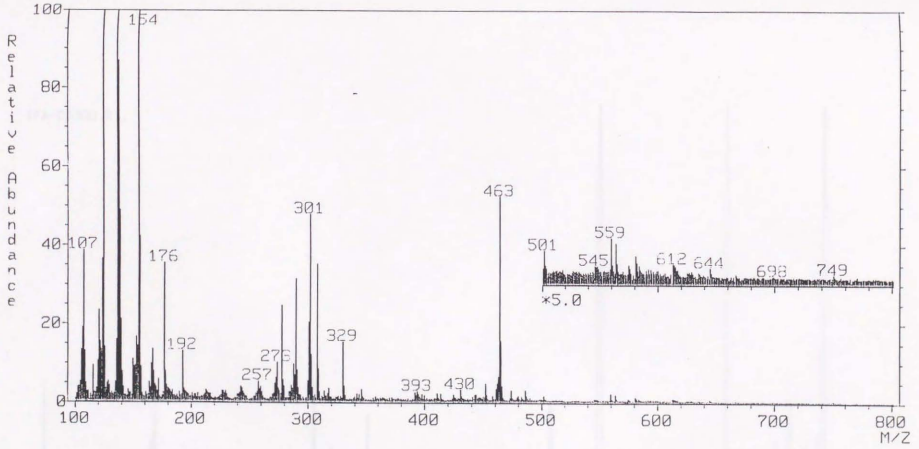


Fig. 17 The FABMA spectrum of 2.

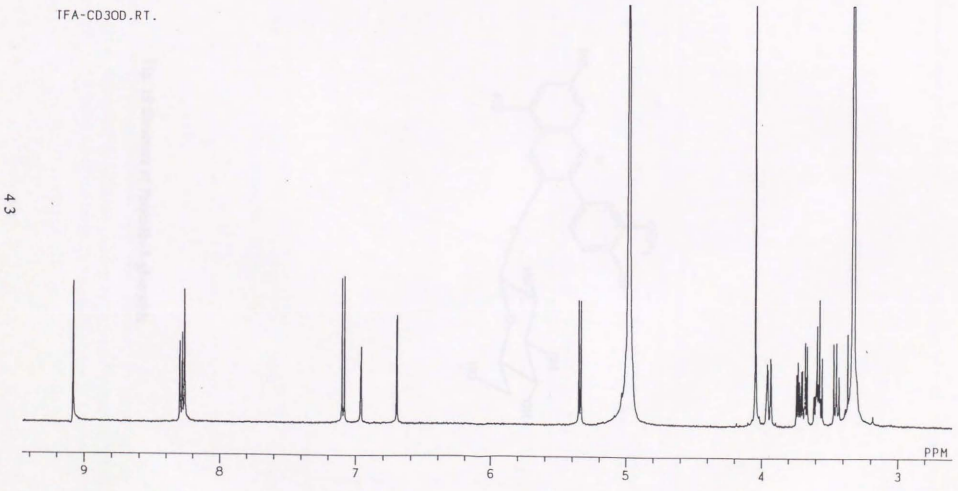


Fig. 18 The 1H-NMR spectrum of 2.

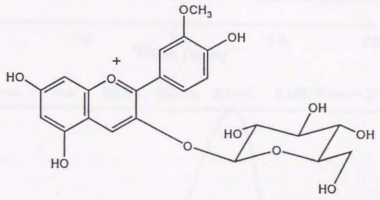


Fig. 19 Structure of Peonidin-3-glucoside.

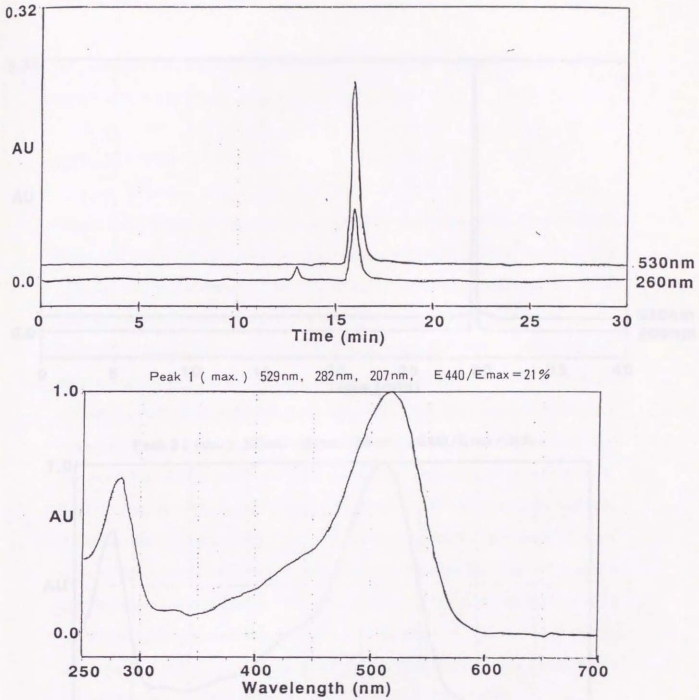


Fig. 20 Chromatograms of the purified peak1 anthocyanin detected with 530 and 260 nm and the UV spectra. Analytical HPLC was carried out using octadecyl silica (ODS) column eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40°C.

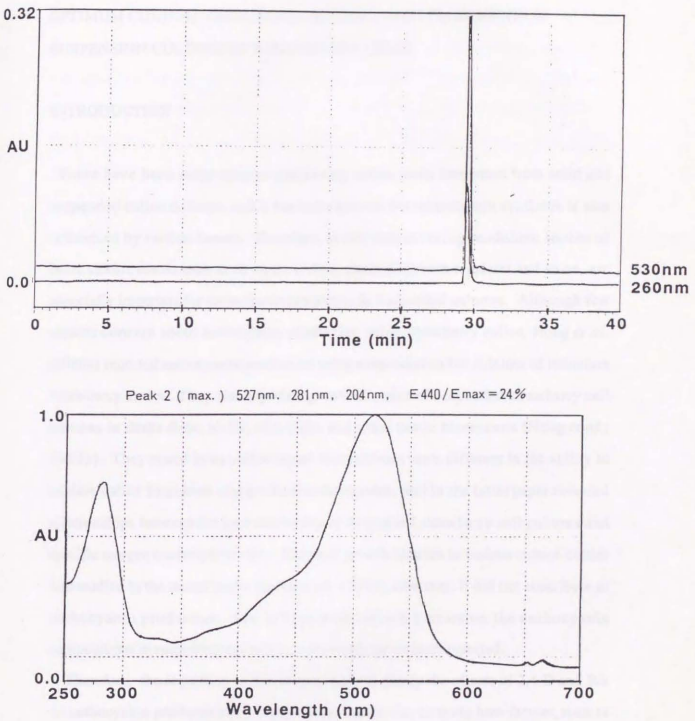


Fig. 21 Chromatograms of the purified peak 2 anthocyanin detected with 530 and 260 nm and the UV spectra. Analytical HPLC was carried out using octadecyl silica (ODS) column eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40°C.

CHAPTER 3

OPTIMUM CULTURE MEDIUM FOR ANTHOCYANIN PRODUCTION IN SUSPENSION CULTURE OF STRAWBERRY CELLS

INTRODUCTION

There have been many articles concerning anthocyanin formation from solid and suspended callus cultures, and it has been known that anthocyanin synthesis is also influenced by various factors. Therefore, to establish culturing conditions, studies of basic culture media such as nitrogen, carbon, mineral, growth regulater and so on, are especially important for anthocyanin production in suspended cultures. Although few reports concern about anthocyanin production using strawberry callus, Hong *et al.* (1989a) reported anthocyanin production using suspended callus cultures of immature strawberry fruits. They also reported growth kinetics of suspended strawberry cell cultures in shake flask, airlift, stirred-jar, and roller bottle bioreactors (Hong *et al.*, 1989b). They stated in an earlier report that cultivars were different in the ability to initiate callus formation and produce anthocyanins, and in the latter paper revealed relationships between the growth kinetics of suspended strawberry cell cultures and specific oxygen consumption rates. Only cell growth kinetics in various culture bottles was studied in the second paper (Hong *et al.*, 1989b), however, it did not contribute to anthocyanin production. The influence of various factors on the anthocyanin compositions in suspended strawberry cultures has never been reported.

Therefore, the objectives of this chapter were to clarify the effects of 2,4-D and BA on anthocyanin production and composition, and further to study how factors, such as the specific ratio of ammonium to nitrate, the total amount of nitrogen, types of sugar, sucrose concentration and combinations of high sucrose in the medium, affect cell growth and anthocyanin synthesis in suspended strawberry cell cultures.

MATERIALS AND METHODS

Auxin and Cytokinin on Anthocyanin Production

To examine the influence of 2,4-D and BA on cell growth and anthocyanin content, calli from leaf (2 g fresh cell weight / 100 mL), which had been sub-cultured three times (1-week intervals) with 3% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks under 800 lux, were cultured with various combinations of 2,4-D (0~5 mg/L) and BA (0~2 mg/L) for two weeks under 8000 lux. After two weeks of incubation, cells were harvested and measured for cell weight and anthocyanin content. The effects of 2,4-D and BA on the major anthocyanin compositions were also studied by measuring the contents of cyanidin-3-glucoside and peonidin-3-glucoside using HPLC.

Cell Suspension Cultures and Basal Culture Media

Suspended cell cultures were the same leaf cells initiated by transferring about 2 g (fresh weight) of friable callus tissue to 100 mL of liquid LS medium supplemented with sucrose at 2%, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks. They were incubated in a rotary shaker (80 rpm) under continuous light intensity of 800 lux at 25 °C. Suspension cultures were grown for 3 weeks, meanwhile the medium was changed every week. The inoculation rate was about 10%. The resulting cell suspension (2 g fresh) was transferred to the freshly prepared various basal liquid medium such as MS, LS and B5 and then incubated under continuous light intensity of 8000 lux at 25 °C on a rotary shaker (80 rpm) to produce anthocyanin production. After 2 weeks of incubation, cells were harvested and measured for cell weight and anthocyanin content.

Effects of Types of Sugar and Sucrose Concentrations

In order to examine the effects of the type of sugar and sucrose concentration on cell growth and anthocyanin production, 2% (W/V) of various sugars (xylose, D-mannose, L-rhamnose, D-arabinose, D-galactose, D-glucose, sucrose, and fructose) were tested, and sucrose concentrations (2, 5, 8, 10 and 12%) were changed further. These cells

were cultured for 2 weeks in B5 medium (Gamborg *et al.*, 1976) under continuous light intensity of 8000 lux.

Ammonium and Nitrate as Nitrogen Source

To examine the influence of nitrogen source on cell growth and anthocyanin content, $(NH_4)2SO4$ and $KNO3$ were added to a nitrogen-free LS medium containing 2% (W/V) sucrose, 2,4-D (1 mg/L), and BA (0.1 mg/L) at a concentrations of 30 mM or 60 mM $NH_4^+ : NO_3^-$ (0:30, 2:28, 4:26, 10:20, 20:10, 30:0, 0:60, 2:58, 10:50, 20:40, 40:20, and 60:0). Cells were cultured for 2 weeks under continuous light intensity of 8000 lux, and were harvested and measured for cell weight and anthocyanin content.

HPLC Analysis of Major Anthocyanins

Solution A (acetic acid : acetonitrile :water-20:25:55), diluted to 35% and containing 0.1% trifluoroacetic acid (TFA) at 4 ℃ was used to extract anthocyanins from fresh callus tissues of leaves to HPLC analysis. The extract obtained was then diluted with 35% solution A to become a concentration of 1 OD (optical density) at 528 nm with a spectrophotometer. After filtration, 2 μL of sample was injected and analyzed with HPLC (Waters 600E) using an octadecyl silica (ODS) column (Develosil ODS-5 4.6 mm ϕ X 250 mm, Nomura Chemical), eluted with 35% solution A diluted with H_2O containing 0.1% TFA and a methanol solution (water: acetic acid: methanol - 80: 15: 5) containing 0.1% TFA at 40 ℃. Chromatograms were recorded on a Shimadzu C-R2A and calculated for percentage of cyanidin-3-glucoside and peonidin-3-glucoside from peak areas.

Determination of Anthocyanins and Cell Growth

Anthocyanins were extracted overnight from fresh callus tissues using a solution containing 0.1% HCl-MeOH at 4 ℃. After centrifugation at 1000 x g for 5 min, the absorbance of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated with the extinction coefficient ($E_{1cm}^{1\%}$=680 at 528 nm) obtained by using

purified peonidin-3-glucoside from cultured strawberry cells as a standard. Major anthocyanins had been identified by FAB-MS and NMR in Chapter 2. Total anthocyanin yield was expressed as mg/100 mL of liquid LS medium in a 500 mL cell culture flask. Cells were separated from the culture medium by filtration through a nylon filter (pore size 30 μm) and weighed. Results were expressed as fresh cell weight per flask.

Statistics

Data was presented as means ± SE (Standard error). Statistical analyses were made using one-way analysis of variance (ANOVA). Differences with P (Probability) < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

2,4-D and BA on Anthocyanin Production in Suspension Culture

There have been a number of reports describing the effects of auxins on anthocyanin formation in various cultured plant cells. In suspended carrot cultures, 2,4-D showed the strongest inhibiting effect on anthocyanin synthesis and had the strongest growth promoting effect (Ozeki and Komamine, 1986), and also inhibited anthocyanin synthesis in *Strobiranthes dyeriana* (Smith *et al.*, 1981). In contrast, 2,4-D stimulated anthocyanins in suspension cultures of *Vitis sp* (Yamakawa *et al.*, 1983b), *Ipomoea batatas* (Nozue *et al.*, 1987), and *Hibiscus sabdariffa* L (Mizukami *et al.*, 1988). Although the reason for this discrepancy with respect to the effects of 2,4-D on anthocyanin synthesis has not been clarified, it seemed that callus cultures of strawberry apparently belong to the latter group (Fig. 11). Therefore the effects of different combinations of 2,4-D and BA were examined (Fig. 22).

The optimum concentration of 2,4-D was found to be 1 mg/L for cell growth in a culture of two weeks (Fig. 22a). Anthocyanin content was strongly affected by 2,4-D concentrations (Fig. 22b). The average anthocyanin content using 2,4-D concentrations

of 0.1, 0.5, 1.0, 2.0, and 5.0 mg/L were 40.5, 68.9, 88.4, 97.2, and 122.3 μg/g of fresh cell respectively, and no anthocyanin accumulations were observed in the cells cultured without 2,4-D, suggesting that anthocyanin content increased as the concentration of 2,4-D increased and the 2,4-D had greater effect on anthocyanin synthesis than BA in cultures two weeks old. Although 5.5 mg/flask of total anthocyanin was produced using concentrations of 1 mg/L of 2,4-D and 0.5 mg/L of BA (Fig. 22c), the anthocyanin production was enhanced when the concentration of 2,4-D was 1 mg/L or higher. The effects of BA on cell growth and anthocyanin content were suppressive. The averaged cell growth using BA concentrations of 0, 0.1, 0.5, 1.0, and 2.0 mg/L were 35.2, 35.1, 32.3, 31.6, and 30.6 g respectively, and the cell growth of both BA concentrations 0 and 0.1 mg/L were significantly ($P< 0.05$) higher than that of BA concentrations 1.0 and 2.0 mg/L. In the early phase of culturing strawberry cells, cell growth was enhanced by increasing the concentration of 2,4-D, and impaired as the concentration of BA rose (Fig. 23a). However, a large amount of anthocyanin content (207 μg/g fresh cell) was obtained in a one-week old culture without adding phytohormone (Fig. 23b). Anthocyanin contents in the cells cultured for one week were clearly impaired as both concentrations of 2,4-D and BA rose, which is contrary to the results shown in Fig. 22. This indicates that anthocyanin accumulation was stimulated by decreasing 2,4-D concentration which had been incorporated into the cell, i,e, not by raising the 2,4-D concentration in the culture medium since these cells were sub-cultured using 2,4-D (1 mg/L) and BA (0.1 mg/L) for three weeks under 800 lux. Therefore, anthocyanin accumulation of the cells was increased as the number of days of the culture growth increased, even when they were cultured in high concentrations of 2,4-D. The cells cultured with relatively high concentrations of 2,4-D were able to produce large amounts of anthocyanin in long-term cultures. This is caused by the stimulation of cell growth by 2,4-D and by stimulation of anthocyanin synthesis. The high productivity in week two was the result of high cell growth. In this experiment, the effects of BA on anthocyanin accumulation and total anthocyanin production in the early phase of culturing strawberry cells were suppressive in the same manner as seen in Fig.

22. The average amount of total anthocyanin using BA concentration of 0.1 mg/L was the highest and significantly ($P< 0.05$) higher than that of BA concentrations 0.5, 1.0, and 2.0 mg/L. However, there were no significant difference between BA concentrations of 0 and 0.1 mg/L. These data led to the best concentration of 2,4-D (0.1 mg/L) and BA (0 or 0.1 mg/L) for anthocyanin production in short term cultures of strawberry cells (Fig. 23c). However, to improve the efficency of anthocyanin production and the safety of callus products for a food ingredient, large cells subcultured with 2,4-D were cultured in the medium without using phytohormones or use of low toxicity phytohormone, e.g. IAA instead of 2,4-D, for anthocyanin production as shown in Chapter 2.

2,4-D and BA on Anthocyanin Compositions in Suspension Culture

Eight types of anthocyanins have been already detected. Two major anthocyanins in the LS medium were identified as cyanidin-3-glucoside and peonidin-3-glucoside (Chapter 2). However, since color of anthocyanin produced from the cultured strawberry cells changes according to the combinations of 2,4-D and BA added to the LS medium, anthocyanin compositions were analyzed using HPLC (Table 6,7). The percentage of cyanidin-3-glucoside increased as the concentrations of both 2,4-D and BA increase (Table 6). The content of cyanidin-3-glucoside in a 2,4-D and BA concentration of 0.5 mg/L and 1 mg/L, respectively, was about seven times greater than that for no phytohormones while that of peonidin-3-glucoside changed opposite way to the results of cyanidin-3-glucoside (Table 7). The total of these two major anthocyanins equals about 80~95% of the total anthocyanins, suggesting that 2,4-D and BA only influence the key enzymes that produce cyanidin and peonidin.

Basal Culture Media

Strawberry cells derived from leaf tissues were cultured in three different liquid basal media (MS, LS, and B5) to examine the influence of medium compositions on cell growth and anthocyanin production. LS and B5 media contained only thiamine as a

vitamin source and low levels of ammonium ion, respectively. MS medium contained high levels of ammonium, four vitamins, and glycine. These medium compositions are shown in Table 8. Cell weight in "Shikinari" cell suspension cultures in each medium was shown (Fig. 24a). Cell weight in both LS and MS media was significantly larger than that of B5 ($P < 0.01$). No difference was found between LS and MS.

Hong *et al.* (1989a) reported on cell growth in liquid suspension using "Brighton" strawberry fruits and four different culture media: MS, LS, B5, and E&R. The report revealed that cell growth in MS medium was slower than that in other media during the first week, but no difference was found among cultures after the second week of incubation. The difference between this results and those of Hong *et al.* may be related to variations in parts of plants and cultivars. Hong *et al.* (1989b) also reported growth kinetics of strawberry cell suspension cultures and revealed specific oxygen consumption rates of strawberry suspension cultures in various culture systems. They showed the oxygen transfer coefficient in the shake flask was higher than in the roller bottle type bioreactor, however the cell growth rate in the shake flask was lower than in the roller bottle. This suggests that the differences of shape in culture bottle and the way of these rotation lead the difference of cell growth. Therefore the difference in the size of flask and the volume of the medium used in our experiments may be the reasons for different cell growth kinetics compared to the results of Hong *et al.*

Anthocyanin content in the B5 medium was highest, reaching about 4-fold higher than in the LS and MS media (Fig 24b). Total anthocyanin production was also highest ($P< 0.05$) in the B5 medium. It was about 2.5-fold higher than those of the LS and MS media (Fig 24c).

There are few reports on cell growth and anthocyanin production in different basal media. The B5 medium yielded the highest anthocyanin production in *Daucus carota* (Nagarajan *et al.*, 1989) and *Euphorbia millii* (Yamamoto *et al.*, 1989). Overall results show that high ammonium in LS and MS stimulated cell growth, but reduced anthocyanin accumulation.

Effects of Sugar Type on Cell Growth and Anthocyanin Production

The effects of eight carbon sources (xylose, D-mannose, L-rhamnose, D-arabinose, D-galactose, D-glucose, sucrose, and fructose) on cell growth and anthocyanin production were studied in the B5 medium containing 2% sugar each. Addition of sucrose resulted in maximum cell yield (Fig. 25a). Cell weight was higher ($P < 0.01$) in the glucose, sucrose, and fructose media than the others. Cell weight increase and anthocyanin production did not occur on xylose, D-mannose, L-rhamnose, and D-arabinose media. These sugars did not support cell growth. Anthocyanin content and total anthocyanins were higher ($P < 0.01$) in the glucose, sucrose, and fructose media than the others. There was no difference among them (Fig.25bc).

Nagarajan *et al.* (1989) reported anthocyanin production from *Daucus carota* using four carbon sources (sucrose, fructose, glucose, and lactose) in suspended cultures. They obtained the maximum cell yield and anthocyanin content using 20 g/L of galactose and ranked the production capacity as galactose, fructose, glucose, sucrose, and lactose in descending order. A comparison of results with our data indicates that strawberry cells are preferable to carrot cells for producing anthocyanins, since they show better growth and anthocyanin accumulation with lower cost carbon sources (sucrose, glucose, and fructose).

Effects of Sugar Type on Anthocyanin Compositions

Figure 26 and Table 9 showed about 8 kinds of anthocyanins were detected with HPLC at 528 nm in callus cultured with sucrose, galactose, glucose, and fructose, respectively. These data suggest that the major anthocyanins (peak 7 and 8) produced from the others sugars were identical to those produced from sucrose. The author had identified the major anthocyanins produced in the strawberry suspension cells cultured in the LS medium containing sucrose as peonidin-3-glucoside and cyanidin-3-glucoside in chapter 2. These results show none of the carbon sources affected the key enzymes for attaching the aglycone to anthocyanidin, resulting in the production of cyanidin-3-glucoside and peonidin-3-glucoside as major pigments. Moreover, pelargonidin-3-

glucoside was not detected from these cells, indicating that the callus cells may lack an enzyme changing dihydrokaempferol into kaempferol. The biosynthetic route of flavonoids and anthocyanins is shown in Fig. 27.

Sucrose Concentrations

The optimum concentrations of sucrose in the B5 medium for cell growth was 2~8% (W/V) and anthocyanin content 5% (Fig. 28). Ten percent sucrose diminished cell growth. Total anthocyanin production using 5% sucrose was also the highest. A high concentration of sugar promotes production of secondary metabolites, such as anthocyanin, in cultured cells. For example, 9.9% sucrose greatly increased anthocyanin production in *Vitis* (Yamakawa *et al.*, 1983), and 5% sucrose in *Populus* (Matsumoto *et al.*, 1973) and *Euphorbia millii* (Yamamoto *et al.*, 1989b). The use of high sucrose concentration to produce anthocyanin was more effective in medium with low ammonium concentrations, such as B5 medium.

Ammonium and Nitrate as Nitrogen Source

In the LS medium containing about 60 mM nitrogen as KNO_3 and NH_4NO_3, the molar ratio of NH_4^+ to NO_3^- was 1: 2, while the B5 medium contained about 30 mM nitrogen, with the ratio of 2:25. The main difference between LS and B5 consisted in total nitrogen concentrations and ratios of $NH_4^+ : NO_3^-$. The B5 medium was the best for anthocyanin production in strawberry cells (Fig.24). It may therefore be possible to increase anthocyanin production in LS medium by controlling the total nitrogen amount and ratio of $NH_4^+ : NO_3^-$ in the LS medium. As shown in Fig. 29 both total nitrogen and ratio of NH_4^+ to NO_3^- had a strong effect on anthocyanin production. Cell growth curve was almost the same between 30 mM and 60 mM total nitrogen (Fig 29a), however, the curve of anthocyanin accumulation was differed in 30 mM total nitrogen compared to that of 60 mM total nitrogen. Anthocyanin content was remarkably peaked at the ratio of 2: 28 (Fig 29b). This tendency was also seen in the total anthocyanin production in the same manner as seen in the anthocyanin content (Fig. 29c). Therefore,

the anthocyanin production was promoted more by 30 mM total nitrogen than by 60 mM. This concentration almost equalled to that in the standard B5 medium. However, the optimal ratio of NH_4^+ to NO_3^- for anthocyanin production was 2 mM :28 mM (Fig.29c), slightly different from that of B5 medium. Anthocyanin production and cell growth were inhibited by high NH_4 (Fig. 29a,b). However, these inhibitions were not attributed to the decrease in the pH value of the culture media, because these pH value were not so different in the culture duration.

Using cultured *Vitis* and *Euphorbia millii*, Yamakawa *et al.* (1983b) and Yamamoto *et al.* (1989) also succeeded in increasing cell growth and anthocyanin production by varying the ratio of NH_4^+ to NO_3^- in the MS and B5 media. Yamakawa *et al.* (1983b) reported that the best ratio of NH_4^+ to NO_3^- was 1 : 1 at 60 mM total nitrogen, while Yamamoto *et al.* (1989) obtained the best cell growth and anthocyanin production at a ratio of NH_4^+ (1): NO_3^- (16) at 30 mM total nitrogen. This suggests that it is necessary to vary the optimum ratio for particular cells to obtain maximum anthocyanin production. In this study, the best nitrogen condition for anthocyanin production was NH_4^+ (2 mM): NO_3^- (28 mM).

Ammonium and Nitrate on Anthocyanins Composition

The author also studied the effect of total nitrogen and the ratio of NH_4^+ to NO_3^- on anthocyanin composition. To compare the composition of anthocyanin produced under different concentrations and the ratios (NH_4^+ NO_3^-) at 2 weeks, the contents of two major anthocyanins, cyanidin-3-glucoside and peonidin-3-glucoside, were measured by HPLC.

The percentage of cyanidin-3-glucoside decreased with increase of NH_4^+ : NO_3^-, while peonidin-3-glucoside increased (Fig. 30). This tendency was clearly seen at 30 mM total nitrogen. The percentage of cyanidin-3-glucoside was higher ($P< 0.01$) at 0 : 30 than at the other ratios. The cyanidin-3-glucoside content gradually decreased with the increase in NH_4^+ : NO_3^- (Fig. 30). Peonidin-3-glucoside content was also higher ($P< 0.01$) at

30 : 0 than at other ratios. Similar effects were seen in 60 mM total nitrogen except 60 :0 (Fig. 31).

Stanko and Bardinskaya (1963) reported that, at the start of formation of anthocyanins, the culture of *Parthenocissus tricuspidata* callus tissues mainly contained derivatives from the group of cyanidin-3,5-diglucoside. However, by the end of growth, and at the start of necrosis, primarily methylated groups of delphinidin-malvidin-3-monoglucoside and malvidin-3,5-diglucoside were observed. Since the nitrogen source affected the cell growth remarkably, the effect of the nitrogen source on the anthocyanin composition in the strawberry suspension culture may be explained by the results by Stanko and Bardinskaya (1963).

The author have reported that 8 kinds of anthocyanins were detected in cultured strawberry cells using HPLC (Fig. 26). Of these, the major anthocyanins were peonidin-3-glucoside and cyanidin-3-glucoside, and the other 6 were minor pigments that accounted for 30% of the total anthocyanins from LS medium. The medium containing NH_4^+ and NO_3^- at 20 : 40 ratio was closest to the composition of LS medium, and the medium at 2 :28 was closest to that of B5 medium. Cyanidin-3-glucoside produced at 2 : 28 was about 2 times greater than that of 20 :40. Therefore cells grown in B5 looked more reddish visually than those grown in LS.

Anthocyanin production in suspended cultures of *Vitis vinifera L.* was increased by changing concentrations of nitrate, ammonium and sugar by Do & Cormier.1991a,b. They demonstrated that high sucrose and low nitrate repress cell growth but enhanced intracellular accumulation of anthocyanins, especially peonidin-3-glucoside. Increasing the ammonium concentrations in the production medium from 2 to 8~16 mM stimulated cell growth and decreased the accumulation of both cyanidin-3-glucoside and peonidin-3-glucoside, while accumulation of peonidin-3-p-coumaroylglucoside was progressively enhanced. Since anthocyanin compositions were monitored with the fixed values of ammonium or nitrate in the medium, comparison of their data with these data (Figs. 30 and 31) are not reliable. A ratio of ammonium and nitrate obviously affects anthocyanin composition in cultured cells. Therefore, by altering the ratio of ammonium and nitrate in

a medium, it is possible to control the color balance of anthocyanins produced in suspended cells.

High Sucrose and Specific Ratio of Nitrogen on Anthocyanins Production

Since anthocyanin production was most active at the ratio of ammonium (2 mM) : nitrate (28 mM) as shown in Fig.29, the effect of high sucrose concentrations on cell growth and anthocyanin accumulation was further studied using a modifid nitrogen source with the LS medium. Cell growth was enhanced and higher ($P < 0.01$) with 5% sucrose, and sucrose concentrations less than 5% depressed cell growth (Fig. 32a). Anthocyanin content was the highest in the 12% sugar concentration. High sucrose levels depressed the cell growth but promoted the anthocyanin accumulation as the sucrose concentration increases (Fig.32b). These data suggests that the range near 5% sucrose is suitable for anthocyanin production (Fig.32c). Total anthocyanin production with 5% sucrose reached about 15 mg /100 mL, which was about 6 fold that with MS medium. However, the cell growth in 2% sucrose was very low although it was the same medium as that where the ratio 2: 28 (ammonium : nitrate) was used (Fig.29). Therefore, the true amount of anthocyanin production using 5% sucrose concentration was greater than the data indicated (Fig.32).

CONCLUSION

1. Suitable hormonal conditions to increase total anthocyanin production changed as the culture time changed. High anthocyanin production in suspended cultures was obtained at the concentrations of BA less than 0.1 mg/L after culturing for one week, and also with the concentrations of 2,4-D higher than 1 mg/L after culturing for two weeks.

2. Anthocyanin composition was influenced by adding 2,4-D and BA to the culture medium. The percentage of cyanidin-3-glucoside increased as the concentrations of both

2,4-D and BA increased, while that of peonidin-3-glucoside changed contrary to the results of cyanidin-3-glucoside.

3. Anthocyanin production was influenced by the basic culture medium, and was higher in the B5 medium than in the LS and MS media.

4. Anthocyanin production was strongly influenced by type of sugar and sucrose concentrations.

5. Major anthocyanin compositions (peonidin-3-glucoside and cyanidin-3-glucoside) were not affected by sugar types when supplementing culture media.

6. The ratio of ammonium and nitrate in the culture medium influenced anthocyanin production. Best results were obtained in a ratio of 2 mM: 28 mM (ammonium: nitrate) at 30 mM total nitrogen. Furthermore, the anthocyanin production increased with the increases in sucrose concentration.

7. The percentage of cyanidin-3-glucoside decreased as the ratio of ammonium : nitrate rose, while peonidin-3-glucoside increased.

Table 6 Effects of 2,4-D and BA on cyanidin-3-glucoside content (%) in suspension cultures of strawberry cells.

(mg/L)		2,4-D					
		0	0.1	0.5	1.0	2.0	5.0
BA	0	7±1.1	17±1.1	20±1.1	17±1.1	17±1.1	15±1.1
	0.1	12±2.3	26±0.5	27±1.1	28±1.7	22±2.3	23±1.7
	0.5	17±1.1	30±1.1	36±1.1	32±0.5	32±1.1	29±2.3
	1.0	19±1.1	30±0	48±1.7	40±1.1	43±0.5	36±0.5
	2.0	21±0.5	31±0.5	37±0.5	39±2.3	38±1.7	32±1.1

Cells (2g fresh weight) were cultured in various concentrations of 2,4-D and BA, and 3% sucrose in LS medium on a rotary shaker (80 rpm) at 25°C for one week. Each value represents the average of three replicates ± standard error of replicates.

Table 7 Effects of 2,4-D and BA on peonidin-3-glucoside content (%) in suspension cultures of strawberry cells.

		2, 4－D					
	(mg/L)	0	0. 1	0. 5	1. 0	2. 0	5. 0
	0	75±2. 8	71±0. 5	71±1. 7	62±1. 1	62±1. 1	63±6. 5
	0. 1	79±2. 3	65±2. 8	68±1. 7	62±1. 1	59±2. 3	60±4. 6
BA	0. 5	74±1. 7	62±1. 1	60±2. 8	59±6. 5	61±0. 5	54±2. 3
	1. 0	73±1. 7	63±1. 7	47±2. 8	53±1. 7	49±2. 3	47±3. 5
	2. 0	67±3. 1	61±1. 7	57±2. 6	56±3. 4	50±5. 7	51±0. 5

Cells (2g fresh weight) were cultured in various concentrations of 2,4-D and BA, and 3% sucrose in LS medium on a rotary shaker (80 rpm) at 25°C for one week. Each value represents the average of three replicates ± standard error of replicates.

Table 8 Composition of MS, LS and B5 media

(mg/L)	MS	LS	B5
NH_4NO_3	1650	1660	–
KNO_3	1900	1900	2500
$(NH4)_2SO_4$	–	–	134
KH_2PO_4	170	170	–
$NaH_2PO_4 \cdot 2H_2O$	–	–	170
H_3BO_3	6.2	6.2	3
$MnSO_4 \cdot 4H_2O$	22.3	22.3	13.2
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	2
KI	0.83	0.83	0.75
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
$CaCl_2 \cdot 2H_2O$	440	440	150
$MgSO_4 \cdot 7H_2O$	370	370	250
$FeSO_4 \cdot 7H_2O$	27.8	27.8	–
Na_2EDTA	37.3	37.3	–
Fe-EDTA	–	–	40
myo-Inositol	100	100	100
Nicotinic acid	0.5	–	1
Pyridoxin HCl	0.1	0.4	10
Glycine	2	–	–

Table 9 Chromatographic mobility of anthocyanins isolated from strawberry suspended callus

Sugar	(A) Peak No. RT (min) 1	2	3	4	5	6	7	8	(B) Peak No. RT (min) 1	2	3	4	5	6	7	8
Sucrose	1.8,	2.2,	2.8,	3.6,	4.1,	6.1,	11.5,	23.2	1.2,	4.0,	4.3,	4.8,	5.0,	6.0,	9.6,	15.1
Galactose	1.7,	nd,	nd,	3.6,	4.1,	6.3,	11.4,	23.5	1.2,	nd,	nd,	4.8,	5.0,	5.9,	9.6,	15.1
Glucose	1.7,	2.2,	2.8,	3.6,	4.0,	6.2,	11.3,	23.4	1.2,	4.0,	4.3,	4.7,	5.0,	6.0,	9.6,	15.1
Fructose	1.8,	nd,	nd,	nd,	4.0,	6.1,	11.6,	23.6	1.2,	nd,	4.3,	4.8,	5.2,	6.1,	9.5,	15.0

(A): Samples were separated with HPLC using 35% solution A containing 0.1% TFA.
(B): Samples were separated using methanol solution containing 0.1% TFA.

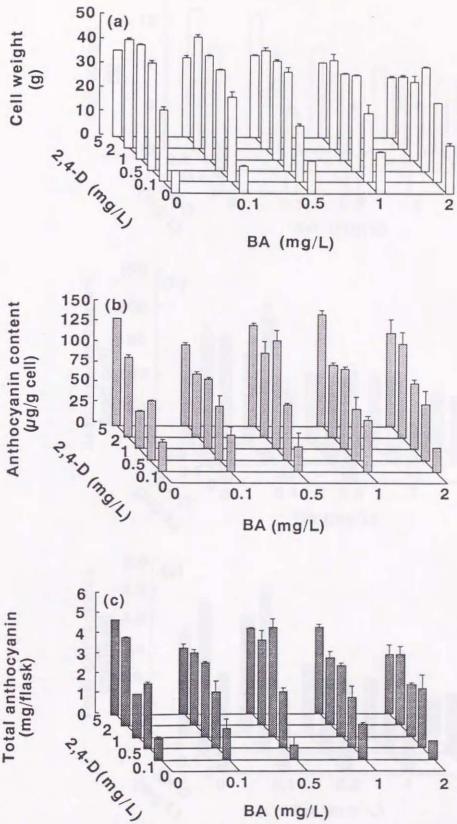


Fig. 22 Influence of various concentrations of 2,4-D and BA on cell growth and anthocyanin production in suspension cultures. Cultures (2g fresh cells) were maintained in LS medium with various concentrations of 2,4-D and BA, and 3% sucrose on a rotary shaker (80 rpm) under light (8000 lux) at 25°C for two weeks. Each value represents the average of three replicates and vertical bar represent standard error of replicates.

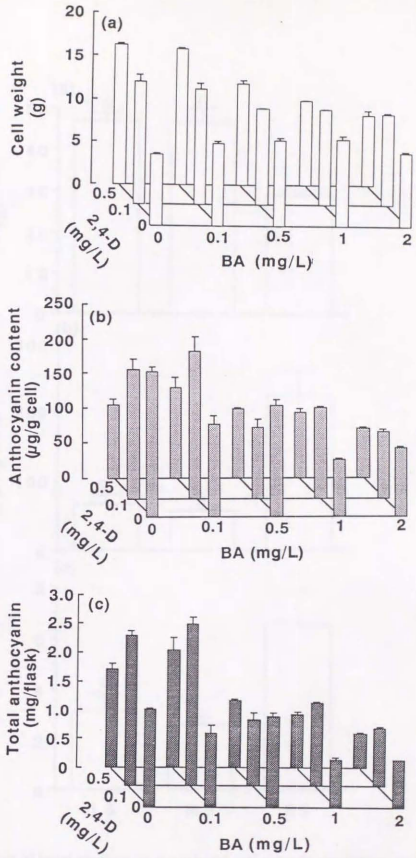


Fig. 23 Influence of 2,4-D and BA on cell growth and anthocyanin production in suspension cultures. Cultures (2g fresh cells) were maintained in LS medium with various concentrations of 2,4-D and BA, and 3% sucrose on a rotary shaker (80 rpm) under light (8000 lux) at 25°C for one week. Each value represents the average of three replicates and vertical bar represent standard error of replicates.

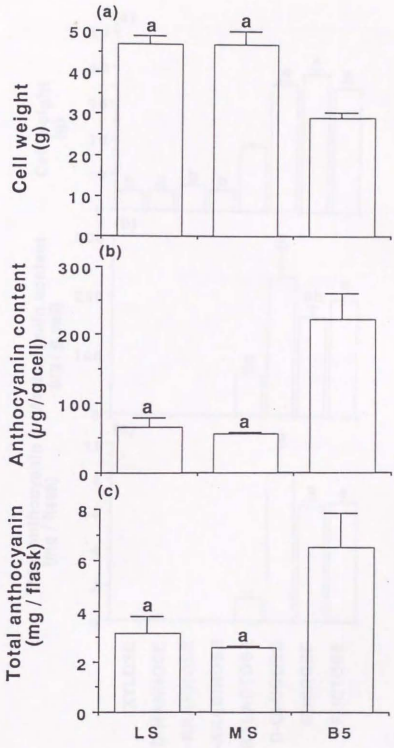


Fig. 24 Effect of basal medium on growth and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained with (1 mg/L) 2,4-D and (0.1 mg/L) BA, and 2% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent the standard error of 3 replicates. a: Bars with same letter do not show significant difference at P= 0.05.

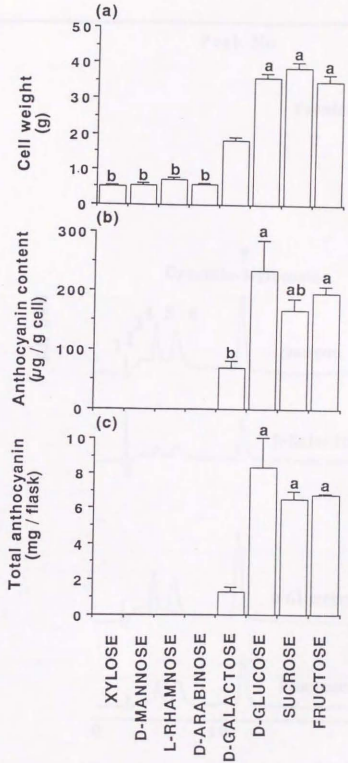


Fig. 25 Effect of various sugar type (2%) on cell weight and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained in B5 medium with (1 mg/L) 2,4-D and (0.1 mg/L) BA on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent the standard error of 3 replicates. a-b: Bars with same letter do not show significant difference at P= 0.05.

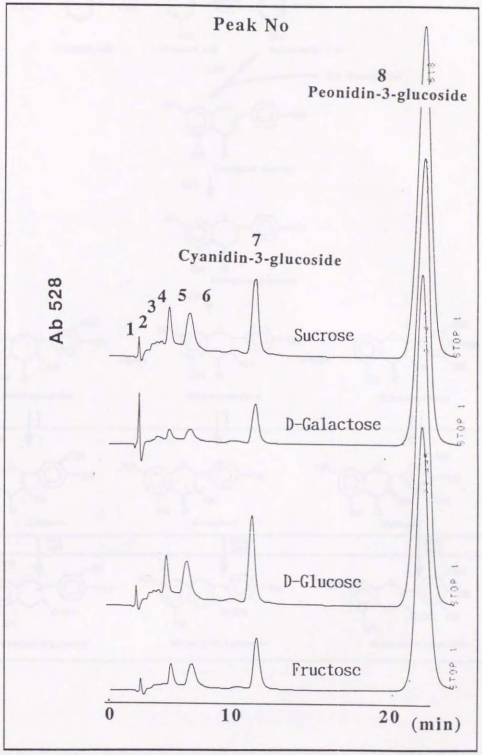


Fig. 26 HPLC profiles of anthocyanins from strawberry cells cltured with various sugars. Samples were separated using 35% solution A containing 0.1% TFA.

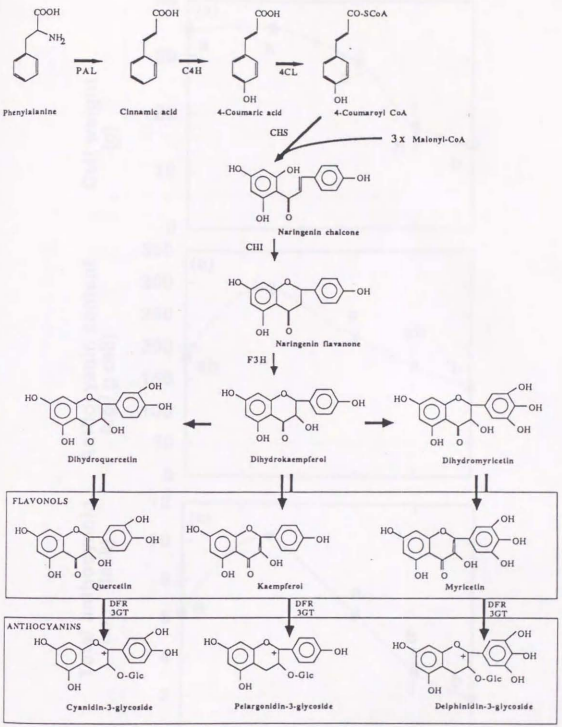


Fig. 27 The biosynthetic route of flavonoids and anthocyanins. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone-3-hydroxylase; DFR, dihydroflavonol-4-reductase; 3GT, UDP-glucose: 3-0-flavonoid glucosyl transferase. Of the compounds shown only the tetrahydroxychalcone and the anthocyanins are pigmented.

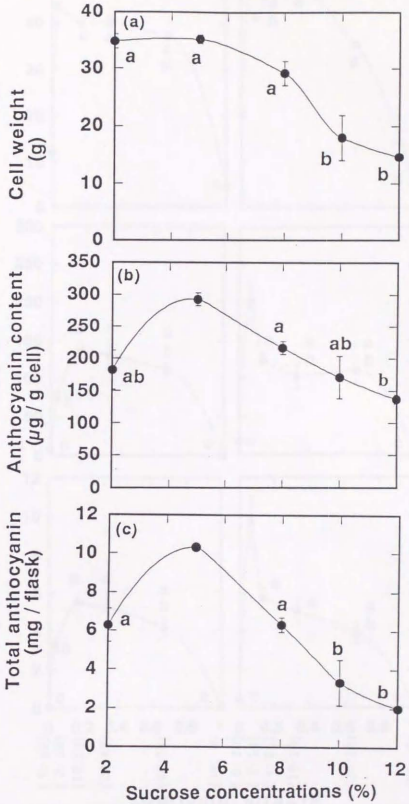


Fig. 28 Effects of sucrose concentrations on cell weight and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained in B5 medium with 2,4-D (1 mg/L) and BA (0.1 mg/L) on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent standard errors for three flasks. The symbols a-b mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

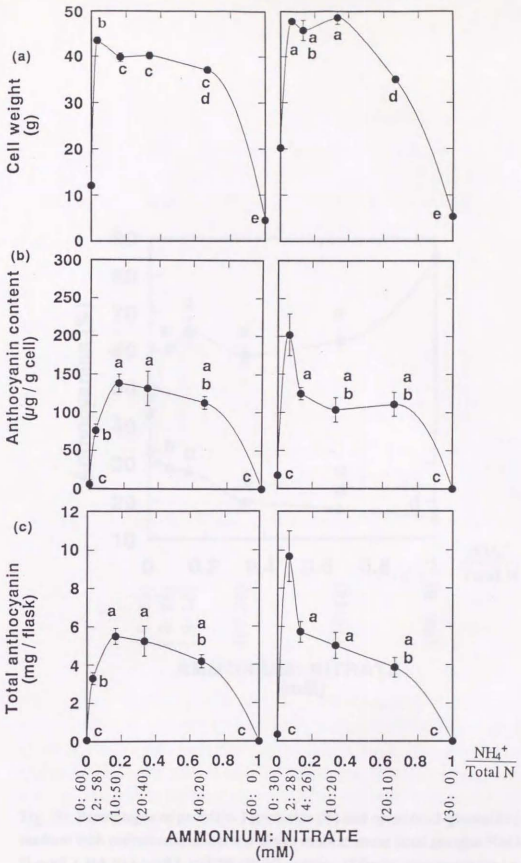


Fig. 29 Effects of ratios of ammonium and nitrate on cell weight and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained in LS medium with various ammonium and nitrate concentrations, 2,4-D (1 mg/L), BA (0.1 mg/L), and 2% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent standard errors for three flasks. The symbols a-e mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

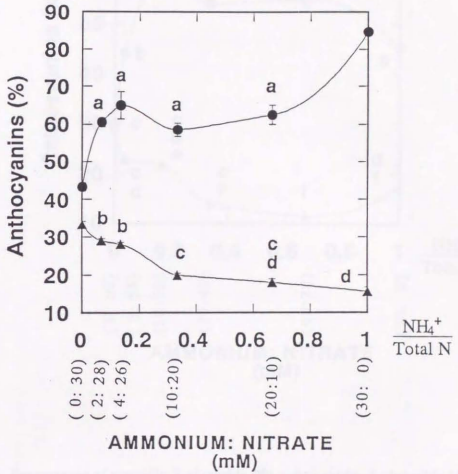


Fig. 30 Percentages of peonidin-3-glucoside (●) and cyanidin-3-glucoside (▲) in LS medium with various ammonium and nitrate concentrations (total nitrogen 30mM), 2,4-D (1 mg/L), BA (0.1 mg/L), and 2% (W/V) sucrose. Cultures were maintained on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent standard errors for three flasks. The symbols a-d mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

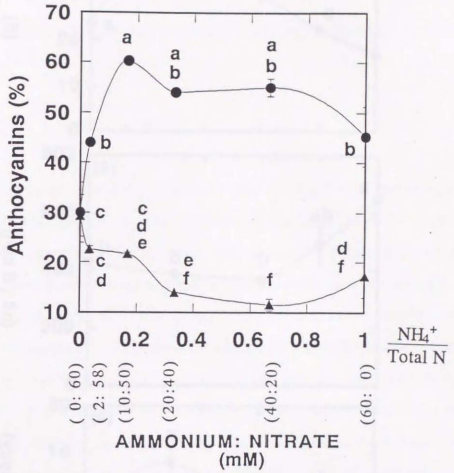


Fig. 31 Percentages of peonidin-3-glucoside (●) and cyanidin-3-glucoside (▲) in LS medium with various ammonium and nitrate concentrations (total nitrogen 60mM), 2,4-D (1 mg/L), BA (0.1 mg/L), and 2% (W/V) sucrose. Cultures were maintained on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent standard errors for three flasks. The symbols a-f mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

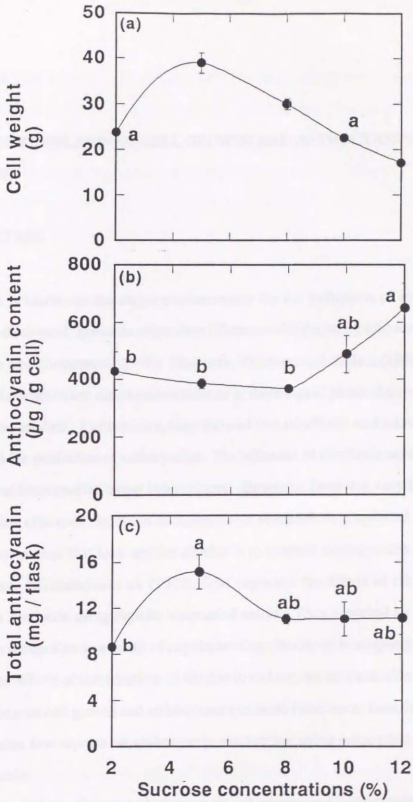


Fig. 32 Effects of sucrose concentrations on cell growth and anthocyanin production of strawberry cells in LS medium with ammonium and nitrate concentrations (2mM: 28mM), 2,4-D (1 mg/L), BA (0.1 mg/L), and various sucrose concentrations. Cultures were maintained on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent standard errors for three flasks. The symbols a-b mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

CHAPTER 4

EFFECTS OF RIBOFLAVIN ON CELL GROWTH AND ANTHOCYANIN SYNTHESIS

INTRODUCTION

Riboflavin is known as the major photoreceptor for the induction of anthocyanin synthesis in duckweed, *Spirodela oligorrhiza* (Thimann and Radner, 1958) and *Sorghum bicolor* (Jain and Guruprasad, 1990). However, Thimann and Radner (1958) revealed that it inhibits or promotes anthocyanin synthesis in these higher plants depending on the concentration applied. Furthermore, they showed that riboflavin and sucrose interact each other in the production of anthocyanins. The influence of riboflavin on anthocyanin synthesis has been studied using intact plants. However, there are very few reports concerning the effects of riboflavin on anthocyanin synthesis in suspended cultures of plant cells, or studies that have applied riboflavin to produce anthocyanins using plant cell cultures. Matsumoto *et al.* (1973) first reported the effect of riboflavin on anthocyanin synthesis using *Populus* suspended callus. They reported an increase in anthocyanin production as a result of supplementing riboflavin in suspended cultures. However, the effects of combinations of riboflavin and sucrose concentration or nitrogen concentrations on cell growth and anthocyanin synthesis have never been investigated. There are also few reports on anthocyanin production using suspended cultures of strawberry cells.

Tests on the effects of sugar and nitrogen on cell growth and anthocyanin production were conducted and reported in Chapter 3. This study explained how the ammonium : nitrate ratio and total nitrogen in the medium affects anthocyanin production and composition in strawberry cell cultures. In spite of these findings, various factors influencing cell growth and anthocyanin synthesis in suspended cultures of strawberry cells remain to be elucidated. Therfore, a study was carried out to examine the effects of

riboflavin on anthocyanin synthesis, cell growth and anthocyanin composition in suspended cultures of strawberry cells. Particularly, the study in this chapter focuses on the effects of riboflavin on anthocyanin production using leaf callus or petiole callus, which are known for low anthocyanin productivity.

MATERIALS AND METHODS

Plant Materials and Callus Formation

Callus tissues were induced from the leaf and petiole of *Fragaria ananassa* cv Shikinari. The tissues were obtained from aseptically regenerated plants, which was described in Chapter 1. The tissues were placed on the LS medium (Linsmaier and Skoog, 1965) containing 3% sucrose, 0.2% Gellangum (Wako Chemical), 0.1 mg/L of BA, and 1 mg/L of 2,4-D. The tissues were then incubated at 25 ℃ under a 16 h-light, 8 h-dark cycle with light intensity 800 lux. Callus tissues were transferred to the freshly prepared LS medium every three weeks.

Cell Suspension Cultures

Suspended cell cultures were initiated by transferring about 2 g (fresh weight) of friable callus tissue to 100 mL of liquid LS medium supplemented with 3% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks. They were incubated on a rotary shaker (80 rpm) under continuous light intensity of 800 lux at 25 ℃. Suspension cultures were grown for 3 weeks, during which the medium was changed every week. The inoculation rate was about 10%. The resulting cell suspension (2 g fresh) was transferred to the freshly prepared various test liquid media and then incubated under continuous light intensity of 8000 lux at 25 ℃ on a rotary shaker (80 rpm) to produce anthocyanin production.

Riboflavin on Petiole Callus

Cell growth and anthocyanin contents from petiole tissue were monitered for 17 days. Effects of riboflavin on cell growth and anthocyanin content were examined. Various riboflavin (Wako) was added at various concentrations (0.2, 1, 4, 8, and 10 mg/L) to the LS medium (100 mL) containing 3% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks, and used for test media after autoclaved for 15 min at 110 °C. Moreover, to examine influences of the combinations of riboflavin and high sucrose concentrations on cell growth and anthocyanin contents, sucrose concentrations (5, 10 and 15%) were also changed.

Riboflavin on leaf callus

In order to examine the effects of riboflavin on cell growth and anthocyanin synthesis using leaf callus, various concentrations of riboflavin (0, 0.2, 1.0, 4.0, 8.0, 10 and 40 mg/L) were added to the modified LS medium (100 mL), which was controled ammonium : nitrate to 2 mM :28 mM using $(NH_4)2SO4$ and $KNO3$ in a nitrogen-free LS medium containing 5% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flask. The K^+ concentration was adjusted to 32 mM by adding KCl. The medium was used after autoclaved for 15 min at 110 °C. Next, to examine the influence of riboflavin (4 mg/L) and nitrogen source on cell growth and anthocyanin synthesis, $(NH_4)2SO4$ and $KNO3$ were added to a nitrogen-free LS medium containing 5% (W/V) sucrose, 2,4-D (1 mg/L), and BA (0.1 mg/L) at a concentrations of 30 mM or 60 mM NH_4^+ : NO_3^- (0:30, 2:28, 4:26, 10:20, 20:10, 30:0, 0:60, 2:58, 10:50, 20:40, 40:20, and 60:0). The K^+ concentration was adjusted to 32 or 62mM by adding KCl when the total nitrogen in the media was 30 or 60 mM, respectively.

Determination of Anthocyanins Content and Cell Growth

Fresh callus tissues for anthocyanins were extracted overnight using a solution containing 0.1% HCl-MeOH at 4 °C. After centrifugation at 1000 x g for 5 min, the absorbance of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated with the extinction coefficient ($E^{1\%}_{1cm}$=680 at 528 nm) which was obtained by

using purified peonidin-3-glucoside from cultured strawberry cells as a standard. Total anthocyanin yield was expressed as mg/100 mL of liquid LS medium in a 500 mL cell culture flask. Cells were separated from the culture medium by filtration through a nylon filter (30 μm) and weighed. Results were expressed as fresh cell weight per flask.

HPLC Analysis of Major Anthocyanins

Solution A (acetic acid : acetonitrile :water-20:25:55), diluted to 35% with water and containing 0.1% trifluoroacetic acid (TFA) at 4 ℃ was used to extract anthocyanins from fresh callus tissues of leaves to allow for HPLC analysis. The extract obtained was then diluted with 35% solution A to become a concentration of 1 OD (optical density) at 528 nm with a spectrophotometer. After filtration, 2 μL of the sample was injected and analyzed with HPLC (Waters 600E) using octadecyl silica (ODS) column (Develosil ODS-5 4.6 mm ϕ X 250 mm, Nomura Chemical), which was eluted with 35% solution A diluted with water containing 0.1% TFA at 40 ℃. The chromatograms were recorded on a Shimadzu C-R2A and calculated for the percentage of cyanidin-3-glucoside and peonidin-3-glucoside contents from the peak areas.

Statistics

Data was presented as means ± SE (Standard error). Statistical analyses were made using one-way analysis of variance (ANOVA). Differences with P (Probability) < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effects of Riboflavin on Petiole Callus

Although riboflavin is known to act as a major photoreceptor for the induction of anthocyanin synthesis under white and UV light, very few reports has focused on suspended callus but intact plant, such as mesocotils of *Sorghum bicolor* (Jain and Guruprasad, 1990) or duckweed, *Spirodela oligorrhiza* (Thiman and Radner, 1958).

Matsumoto *et al.* (1973) reported increase of total anthocyanin production by supplement of riboflavin using *Populus* suspended callus culture. They succeeded in increasing 22% total anthocyanin production using LS medium containing riboflavin (0.25 mg/L) and sucrose (3%) after 6 days culture. Therefore, the author tried to increase anthocyanin production in suspended callus, which has low anthocyanin productivity, using riboflavin. The author has already reported that cell growth and anthocyanin production were compared among calli derived from apical meristem, leaf and petiole tissues at Chapter 2. It was observed that callus cultured from the petiole was considerably less effective than other tissue parts in terms of cell growth and pigment formation (65.6 μg/g cell) at two weeks (Fig. 10). Almost the same anthocyanin production was obtained in the control at day 14 (Fig. 33).

Concentrations of riboflavin strongly affected cell growth (Fig. 33a). Riboflavin remarkably reduced cell growth, and significant differences ($P< 0.05$) in cell growth were observed after eight days. There was no significant difference between the control media and the medium having the riboflavin concentration of 0.2 mg/L regardless of the length of culture duration. However, cell growth rates were reduced depending on the concentration of riboflavin; especially for concentrations of 8 and 10 mg/L, cell growth was nearly inhibited, indicating that high concentrations of riboflavin have the negative effect on cell growth in suspended strawberry cultures.

Anthocyanin synthesis was stimulated remarkably by riboflavin (Fig. 33b). The highest content (176 μg/g cell) was obtained at eight days in a solution with a riboflavin concentration of 4 mg/L, which was 3.2 times greater than that in the control media. Anthocyanin contents produced at the concentrations of 4~8 mg/L of riboflavin were significantly higher ($P< 0.05$) than that produced in the control media at eight days. Moreover, although maximum anthocyanin content was obtained after about two weeks in the control, all anthocyanin-content peaks in the mediums supplemented with riboflavin occurred on day eight, suggesting a riboflavin has a promoting effect to shift the peak of anthocyanin synthesis at about week one. Further, the maximum anthocyanin content in the medium with a riboflavin concentration of 4 mg/L was about

2.9 times greater than that in the control media at two weeks; however, the anthocyanin contents of the media supplemented with riboflavin were drastically reduced after two weeks. The results show that anthocyanin synthesis was markedly enhanced by supplementing riboflavin in the media, and the duration required for the strawberry cell cultures to obtain the maximum anthocyanin content was reduced to nearly half of that for cultures grown in the control medium. This result may indicate that riboflavin has a positive effect on anthocyanin synthesis for a period of only one week in this culturing condition due to the degradation of riboflavin by light irradiation (Song and Metzler, 1967).

Riboflavin has been shown to both inhibit and promote the synthesis of anthocyanin in *Spirodela oligorrhiza* depending on the concentration used (Thiman and Radner, 1958). It inhibited pigment formation when concentrations were above 37.6 mg/L. The promoting effect of riboflavin for anthocyanin production in *Spirodela oligorrhiza* was clarified in seven days and increased in 10 days with a concentration of 22.6 mg/L On the other hand, in suspended strawberry-cells cultures, riboflavin concentrations of 4~8 mg/L were best for stimulating anthocyanin synthesis (Fig. 33b). The differences between our results concerning effective concentrations of riboflavin for anthocyanin production in cultures of strawberry and *Spirodela oligorrhiza* may be related to species variations and differences related to intact plants or suspended cells.

Total anthocyanin production is shown in Fig. 33c. Total anthocyanin using a riboflavin concentration of 4 mg/L reached about 1.3 mg/flask, 2.5 times greater than that produced in the control media at day eight. This value was almost equal to that in the control media after 14 days. A high riboflavin concentration of 10 mg/L produced low total anthocyanins regardless of the culturing duration. The reduction of cell growth and enhancement of anthocyanin synthesis as a result of riboflavin supplementation revealed two types of total anthocyanin production (Fig. 33c). The maximum total anthocyanin production that peaked after two weeks was obtained using riboflavin concentrations of 0, 0.2 and 1.0 mg/L, while the maximum total production was obtained after one week using concentrations of 4.0, 8.0 and 10 mg/L (Fig. 33c).

Furthermore, the total production obtained after one week using a concentration of 4 mg/L was almost equal to that obtained from the control medium after two weeks. Therefore, there is a possibility that culturing time can be reduced by supplementing the medium with a riboflavin concentration in the range of 4 mg/L in order to improve anthocyanin productivity in the suspended cultures of strawberry cell. This effective concentration of riboflavin was completely higher than that of Matsumoto *et al.* (1973). These may be due to the difference of plant species.

Sucrose and Riboflavin Effects on Petiole Callus

Thimann and Radner (1958) reported anthocyanin formation using intact plant of duckweed, *Spirodela oligorrhiza.* They reported that a combination of riboflavin (22.6 mg/L) and sucrose (0.85%) approximately doubled anthocyanin formation. On the other hand in suspended cultures of plant cells, a high concentration of sugar also promotes the production of anthocyanins. For example, a sucrose concentration of 9.9% greatly enhanced anthocyanin production in *Vitis* (Yamakawa *et al.*, 1983), and a 5% sucrose concentration for *Populus* (Matsumoto *et al.*, 1973) and *Euphorbia milli* (Yamamoto *et al.*, 1989).

Therefore, the author tested combinations of sucrose with the best concentration of riboflavin (Fig. 33) in an attempt to further improve the productivity of anthocyanin in suspended strawberry-cell cultures. The results of high sucrose concentrations and riboflavin on cell growth and anthocyanin synthesis were shown in Fig. 34. Combinations of high sucrose concentrations and riboflavin markedly suppressed cell growth in suspended strawberry cell cultures. The average cell weight in media with a riboflavin concentration of 4 mg/L and sucrose concentrations of 5, 10 and 15 mg/L at 17 days were 9.5, 7.3 and 5.1 g/flask, respectively. These weights were only about 32, 24 and 17%, respectively, as compared to the cell weight obtained in the control media. This reduction of cell growth was assumed to a result of high osmotic pressure owing to high sucrose concentrations and inhibited cell growth caused by riboflavin.

However, anthocyanin synthesis was strongly promoted by high concentrations of sucrose and riboflavin (Fig. 34b). A combination of riboflavin (4 mg/L) and sucrose (10%) induced the highest anthocyanin content. Furthermore, 5 and 10% concentrations of sucrose induced anthocyanin contents that were significantly higher than that of the control media at eight days ($P< 0.05$). These values were 582 and 703 μg/g cell and almost 10.6 and 12 times greater, respectively, than the content obtained in the control media. This was almost 3.3 and 4.0 times greater than the supplementation of riboflavin + 3% sucrose (Fig. 33b).

Total anthocyanin production was also influenced by the riboflavin and sucrose supplements (Fig. 34c). The total anthocyanin obtained with sucrose supplements of 5 and 10% reached 6.7 and 7.3 times, respectively, that obtained in the control media on day eight. Furthermore, the amounts were 2.5 and 2.8 times that obtained in the control media after maximum production of 14 days. Compared to the case of riboflavin + 3% sucrose, approximately 3 times more anthocyanin was obtained from the medium supplemented with a 10% concentration of sucrose after eight days (Fig. 34c). This result suggested that a combination of sucrose and riboflavin was capable of reducing the culturing time and enhancing the production of anthocyanin in suspended strawberry-cell cultures.

Riboflavin on Leaf Callus in Optimun Medium

The author have already reported that anthocyanin production is most active in suspended strawberry cell cultures grown in modified LS medium containing 5% sucrose (W/V) and an ammonium: nitrate ratio of 2: 28. The total anthocyanin was about 15 mg/100 mL of culture medium, nearly 6 times greater than that in MS medium (Fig. 32). More enhancement in anthocyanin production and possibility to shorten culture duration were predicted from the results of Figs, 33 and 34 when leaf callus was cultured in this modified medium supplmented by riboflavin. Therefore, the effect of various concentrations of riboflavin on the production of anthocyanin in cultures after a week was investigated (Fig. 35). High concentrations of riboflavin stagnated cell growth as

shown in Fig. 33a, with concentrations higher than 4 mg/L significantly ($P< 0.05$) depressing cell growth as compared to the control (Fig. 35a). Cell growth using the riboflavin concentration of 8 mg/L was only 33% as compared to that of the control. However, the anthocyanin synthesis was strongly enhanced by riboflavin (Fig. 35b). The highest anthocyanin contents (about 1000 μg/g cell) were obtained using riboflavin concentrations of 8 and 10 mg/L, both being significantly ($P< 0.05$) higher than that of the control. The induction ability ranked as 8.0, 10, 40, 4.0 and 1.0, and the others in descending order at one week. The media used in this experiment included modified LS medium adjusted to an ammonium: nitrate ratio of 2 mM: 28 mM and containing 5% sucrose for optimal anthocyanin production (Fig. 32), and the same 5% (W/V) sucrose added to the LS and B5 media as a control. An anthocyanin content approximately 8.3 times greater than that obtained in the LS medium was achieved in the 8 and 10 mg/L concentrations, showing that anthocyanin synthesis is strongly enhanced by altering the ammonium: nitrate ratio and riboflavin supplements.

Although the highest total anthocyanin production was obtained using a riboflavin concentration of 1 mg/L, there was no significant difference ($P< 0.05$) among the riboflavin concentrations of 0, 1.0 and 4.0 (Fig. 35c). This result shows that the riboflavin promotes anthocyanin synthesis, but this promotion does not result in a significant increase in total anthocyanin production due to the depression of cell growth by riboflavin.

The cell growth caused stagnation by riboflavin continued for two weeks (Fig. 36a). The same inhibition pattern on cell growth by riboflavin was observed as in Fig. 35a. The riboflavin concentration of 4 mg/L produced the highest anthocyanin content, which was 1.5 times greater than the control (Fig. 36b). This value was almost equal to that obtained after one week, however, the increased anthocyanin contents obtained in the concentrations of 8.0, 10 and 40 mg/L after one week decreased drastically to half or less after two weeks. The highest total anthocyanin production (about 19 mg/flask) was obtained using a riboflavin concentration of 4 mg/L. This is not significant among that of riboflavin concentrations of 0, 0.2, 1.0 and 4.0, suggesting that riboflavin promotes

anthocyanin synthesis but is not effective to increase total anthocyanin production in the optimal medium at both one and two weeks on the contrary to the results of anthocyanin production using petiole callus. The difference of the result of Matsumoto *et al.* (1972) may be related to the variations of plants and cultivars.

Effects of riboflavin and nitrogen source

Although the author could not obtain good results by supplementing riboflavin in the optimal culture media to increase total anthocyanin production in the experiment (Figs. 35 and 36), the difference between the results and experiments in Figs. 33 and 34 is due to origin of callus and composition of culture media. A callus from petiole has lowest anthocyanin productivity among the calli from various different parts of strawberry. This may express that there is some physiological difference in the anthocyanin synthesis among them. It is obvious that the culture medium greatly influence cell growth and production of secondary metabolites. Yamakawa *et al.* (1983b) and Yamamoto *et al.* (1989) also reported changes in anthocyanin productivity by varying the ratio of NH_4^+ and NO_3^- using cultured *Vitis* and *Euphorbia milli*. Of strawberry suspension cells, both cell growth and anthocyanin synthesis were strongly influenced by basal culture media such as LS, MS and B5 (Hong *et al.*, 1989a), suggesting the physiological pathway in cells might be altered by changing the total nitrogen and the molar ratio of NH_4^+ to NO_3^-. Therefore, the effects of riboflavin on anthocyanin synthesis were further investigated by using leaf callus cultured in 12 molar ratios of NH_4^+: NO_3^-. Considering a suitable riboflavin concentration for cell growth and anthocyanin synthesis from results of Figs. 35 and 36 a riboflavin concentration of 4 mg/L was used for investigations. Cell growth and anthocyanin production were studied using various ratios of ammonium and nitrate (Fig. 37). Cell growth was highly influenced by the ratio of ammonium and nitrate as shown in Fig. 29, and nearly the same results were obtained in this experiment (Fig. 37a). Cell growth was stalled markedly with ammonium: nitrate ratios of 0: 60, 60: 0, 0: 30 and 30: 0. The highest anthocyanin content (853 μg/g cell) was obtained in a ammonium : nitrate ratio of 0:30 (Fig. 37b). This was significantly higher ($P< 0.05$)

than that of the ratios of 2: 28 and 20: 40, the typical nitrogen ratios used in B5 and LS. The average anthocyanin content in the ratios of 0: 60, 60: 0, 0: 30 and 30: 0 were 574, 608, 853 and 694 μg/g cell, respectively. Although the highest total anthocyanin production (10 mg/flask) was obtained using the ratio of 4: 26, there was no significant difference among the ratios of 2: 28, 4: 26 and 10: 20 (Fig. 37c) in terms of total anthocyanin production. Total anthocyanin production in the ratios of 0: 60, 60: 0, 0: 30 and 30: 0 were 3.8, 2.8, 6.2 and 3.4 mg/flask, respectively, after one week. This was relatively greater when the total amount of nitrogen was 30 mM. Cell growth after two weeks is shown in Fig. 38a. Cell growth patterns of the various ratios of ammonium and nitrate (Fig. 38a) were nearly identical to those obtained in Fig 29a. Most cell growth was 1.4 to 2.0 times as large as that measured after one week, however, even negative growth rates were observed in the ratios of 60: 0 and 30: 0. Anthocyanin content changed drastically after two weeks. Anthocyanin synthesis was decreased markedly after 2 weeks in all samples (Fig. 38b), especially the content in the ratios of 0: 60, 60: 0, 0: 30 and 30: 0 fell by 71, 74, 56 and 75%, respectively, as compared with that measured after one week. However, nearly the same or lower content as compared with that after one week was obtained in the ratios of 10: 50, 20: 40, 2:28, 4: 26 and 10: 20. Total anthocyanin production also changed drastically after 2 weeks as compared with that after 1 week. Total anthocyanin production increased 48, 68, 87, 81, 50 and 39% in the ratios of ammonium and nitrate adjusted to 2: 58, 10: 50, 20: 40, 2: 28, 4:26 and 10: 20, respectively (Fig. 38c). On the contrary, total anthocyanin production decreased in the ratios of 0: 60, 40: 20, 60: 0, 0: 30, 20: 10 and 30: 0. In view of this fact the total anthocyanin production obtained after one week was 2.5, 3.9, 1.5 and 4.5 times greater than that after two weeks in the ratios of 0: 60, 60: 0, 0:30 and 30: 0, respectively.

Considering the effects of riboflavin, total anthocyanin production increased with culture durations when cells were cultured in favorable ratios of ammonium and nitrate. However, the anthocyanin content obtained after one week was nearly the same or increased only slightly as compared with that measured after two weeks, suggesting that

riboflavin does not contribute to an increase in total anthocyanin production in favorable culture media as observed in Figs. 35c and 36c. Therefore, riboflavin has a positive effect on total anthocyanin production for a period of only one week when callus is cultured under unfavorable conditions, such as ammonium : nitrate ratios of 0: 60, 60: 0, 0: 30 and 30: 0.

However, the enhancement of anthocyanin synthesis in the early stages of culturing may not be related to riboflavin, because there was no control (absence of riboflavin) in this experiment, and the effects of various ratios of ammonium and nitrate on cell growth and anthocyanin synthesis after two weeks using suspended strawberry cell cultures have only been studied in Fig. 29.

Comparisons of riboflavin supplements (4 mg/L) and control on cell growth and anthocyanin production at week one and two in ammonium: nitrate ratios of 0: 60, 40: 20, 60: 0, 0: 30, 20: 10 and 30: 0 were made (Figs. 39 and 40). Riboflavin inhibited cell growth regardless of the culture duration (Figs. 39a, 40a). Cell growth in the ammonium : nitrate ratios of 40: 20 and 20: 10 were reduced to 37 and 47% after one week (Fig. 39a), and 19 and 40%, respectively after two weeks (Fig. 40a).

Anthocyanin content was also influenced by riboflavin. Anthocyanin content was 7.1 times higher at the ratio of 30:0 as compared to the control after one week (Fig. 39b). In all media supplemented with riboflavin, anthocyanin synthesis was greatly enhanced as compared to that of the control for both one- and two-week culture durations (Figs. 39b, 40b). However, the anthocyanin contents produced in solutions with riboflavin after one week were remarkably higher than that produced after two weeks, except for the ratio of 40: 20. The author cannot explain the reason why the greater anthocyanin content was observed in this experiment at the ratio of 40: 20. However, this tendency provided nearly the same results illustrated in Figs. 37 and 38. Although total anthocyanin production expressed the same tendency as the results recorded in Figs. 37 and 38, about 10, 2.6, 2.0 and 1.6 times greater total anthocyanin than the control was obtained in ammonium: nitrate ratio of 30: 0, 60: 0, 0: 60 and 0:30 after 1 week, respectively (Fig. 39c). These values were significantly higher ($P< 0.05$) than that measured in the control.

Furthermore, the total anthocyanin in one-week cultures supplemented with riboflavin was greater than that produced after two weeks (Fig. 40c). Although these values were somewhat greater than that obtained in Figs. 37 and 38, remarkable improvements in anthocyanin synthesis and total anthocyanin production as a result of riboflavin supplements were observed as a whole, suggesting that riboflavin operates to increase anthocyanin synthesis in unfavorable medium conditions only during early stages of culturing. The similer results were observed in the LS culture medium using both calli from leaf and apical meristems, which reduce the potentiality of anthocyanin formation (Data not shown). These data demonstrates that physiological pathway in synthesizing anthocyanin is different in callus types and condition on the callus, and this is easily influenced by culture medium even if the callus had a constant potentiality of anthocyanin synthesis.

It is known that plants and microorganisms are capable of synthesizing riboflavin in their cells. Strawberry also contains riboflavin, ranging from 6~29 μg/100 g fresh weight (Hudson and Mazur, 1985). Klein (1959) observed that exogenously-added riboflavin plus blue light enhanced phytochrome-mediated opening in the excised hook of beans. The enhancement could be obtained even by passing blue light through a solution of riboflavin placed directly above the hooks, implying that the improvement is due to the excitation of phytochrome caused by the fluorescent emission of riboflavin. Song *et al.* (1981) also reported that phytochrome complexed with endogenous flavin can be isolated from etiolated oat seedling, and Pr to Pfr phototransformation by blue light became higher in the presence of riboflavin (Sarkar and Song, 1982). On the other hand, another report expressed that phytochrome controls anthocyanin synthesis in tomato seedlings (Drumm and Mohr, 1981). Therefore, it is obvious that anthocyanin synthesis is influenced by riboflavin. While, although Thimann and Radner (1958) reported anthocyanin synthesis in the dark using *Spirodela oligorrhiza*, anthocyanin was not synthesized in the dark even when suspended strawberry cells were cultured in riboflavin solution (data not shown). Considering the effects of riboflavin on anthocyanin synthesis in unfavorable culturing conditions such as ammonium : nitrate ratios of 0: 60,

60: 0, 0: 30 and 30:0, the enzymatic pathway of producing phytochrome or riboflavin in strawberry cells might be damaged when cultured in the above media. Therefore anthocyanin synthesis might be enhanced by supplementing the culture media with riboflavin.

By the use of riboflavin, reduction in culturing time and increase in anthocyanin productivity will be possible even when a callus having low anthocyanin productivity is used for experiments.

Effects of riboflavin on anthocyanin composition

Changes in anthocyanin compositions as a result of various factors, such as culture durations (Stanko and Bardinskaya, 1963), sucrose concentrations and nitrogen source (Do and Cormier, 1991a,b), white light (Stafford, 1965; Drumm and Mohr, 1978), types of light (Jain and Guruprasad, 1990) have been previously reported. This anthocyanin composition in suspended strawberry cell cultures is also shown to be greatly influenced by altering ammonium and nitrate ratio in a culture medium in Chapter 3. Therefore, compositions of major anthocyanins were investigated using HPLC whethere or not the riboflavin acted on the composition.

The color of cells cultured in solutions supplemented with riboflavin changed according to the concentration of riboflavin and culture duration. The compositions of major anthocyanins were investigated using HPLC.

Peonidin-3-glucoside produced in the medium with a riboflavin concentration of 8 mg/L was significantly higher ($P< 0.05$) after one week, while cyanidin-3-glucoside was significantly ($P< 0.05$) lower than that measured in the control (Fig. 41). However, there was no significant difference ($P< 0.05$) in either peonidin-3-glucoside or cyanidin-3-glucoside produced in solutions with riboflavin concentrations of 0, 0.2 and 1 mg/L after a one-week culture duration. Although a significant difference ($P< 0.05$) was not observed in either peonidin-3-glucoside or cyanidin-3-glucoside with these riboflavin concentrations after a two week culture duration, there was a tendency for increased peonidin-3-glucoside as riboflavin concentration rose. On the other hand, the amount of

cyanidin-3-glucoside was slightly lower or constant through culture duration (Figs. 41 and 42). These data revealed that peonidin-3-glucoside obviously increased as the concentration of riboflavin increased, while the amount of cyanidin-3-glucoside fell or remained constant, however, both absolute percentages of peonidin-3-glucoside and cyanidin-3-glucoside fell according to culture duration, suggesting an increase in the rest of minor 6 anthocyanins as culture duration increased.

White light induces the formation of cyanidin-3-glucoside in *Sorghum* mesocotyls (Stafford, 1965; Drumm and Mohr, 1978), whereas Jain and Guruprasad (1990) showed blue light (450 nm) promoted the synthesis of apigeninidin. In this experiment, the amount of cyanidin-3-glucoside decreased according to culture duration contrary to the results of Stafford (1965) and Drumm and Mohr (1978), and in spite of the fact that the cells were cultured under fluorescent light. I can not explain this, however, it may be related to the results of Stanko and Bardinskaya (1963) showing that cyanidin-3,5-diglucoside was mainly contained in the start of *Parthenocissus tricuspidata* callus, and primarily methylated groups of delphinidin-malvidin-3-monoglucoside and malvidin-3,5-diglucoside were present by the end of growth. Although there is no report concerning the effect of riboflavin on anthocyanin compositions, riboflavin is the major photoreceptor for the induction of anthocyanin synthesis in *Sorghum bicolor* using white and UV-A light (Jain and Guruprasad, 1990). Therefore, riboflavin may promote the metabolism of the shikimic acid channel for producing anthocyanin, enabling peonidin-3-glucoside to increase with the rise in riboflavin concentration, or culture duration may be shortened by half to obtain the maximum anthocyanin production as observed in Figs. 39 and 40.

CONCLUSION

1. Riboflavin inhibited cell growth but stimulated anthocyanin synthesis in suspended strawberry cell cultures.

2. A high sucrose concentration enhances anthocyanin synthesis, however drastic improvements in reducing culturing time for anthocyanin production were observed only when petiole callus or leaf and apical meristem were used, which reduces the potential of anthocyanin formation.

3. Riboflavin did not contribute to an increase in total anthocyanin production in the modified optimum medium using leaf callus.

4. Riboflavin promoted anthocyanin synthesis to maximize the production after one week only when leaf callus was cultured in unfavorable media, such as ammoniun : nitrate (0 : 60, 60 : 0, 0 : 30, 30 : 0).

5. Peonidin-3-glucoside increased as the concentration of riboflavin increased, while the amount of cyanidin-3-glucoside decreased or remained constant. However, both of their absolute percentages fell according to the culture duration when the riboflavin-supplemented culture media was used.

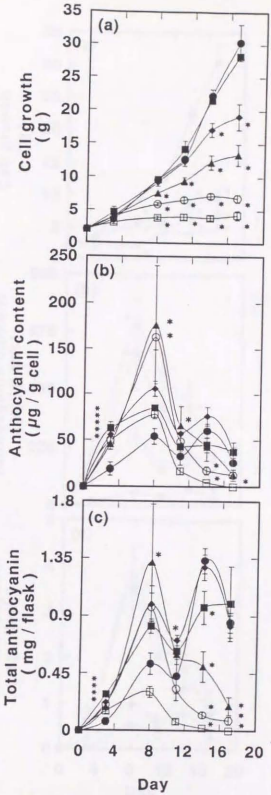


Fig. 33 Effects of riboflavin on cell growth and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained with (1 mg/L) 2,4-D and (0.1 mg/L) BA, and 3% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 17 days. Concentrations of riboflavin ●: 0 mg/L ; ■ : 0.2 mg/L ; ◆ : 1 mg/L ; ▲ :4 mg/L ; ○ : 8mg/L ; □ : 10 mg/L. Each value represents the averages of three replicates, vertical lines represent standard error of replicates. The symbols * mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

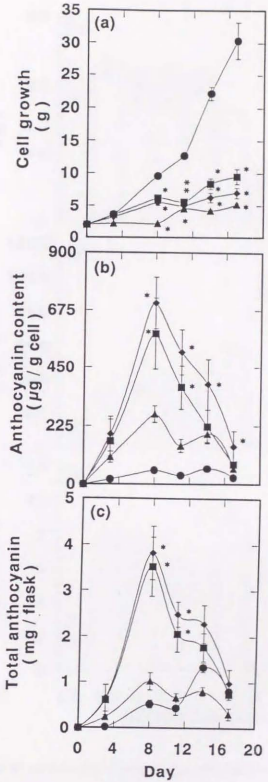


Fig. 34 Effects of riboflavin (4 mg/L) and various sucrose concentrations on cell growth and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained with (1 mg/L) 2,4-D and (0.1 mg/L) BA, and 3% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 17 days. Riboflavin 4 mg/L + sucrose % ●: Control, riboflavin 0 + sucrose 3% ; ■ : 5% ; ◆ : 10% ; ▲ :15%. Each value represents the averages of three replicates, vertical lines represent standard error of replicates. The symbols * mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

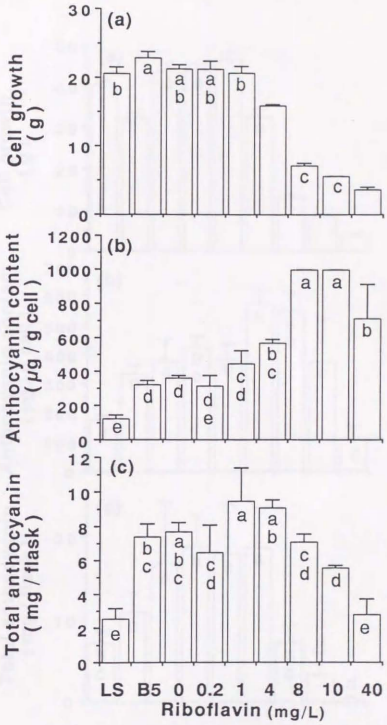


Fig. 35 Effect of various riboflavin concentrations (0~40 mg/L) on cell growth and anthocyanin synthesis in suspension cultures of strawberry cells. Cultures were maintained in LS medium with modified ammonium and nitrate concentrations (2mM: 28mM), 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 1 week. Vertical bars represent the standard error of 3 replicates. a-e: Bars with same letter do not show significant difference at $P = 0.05$.

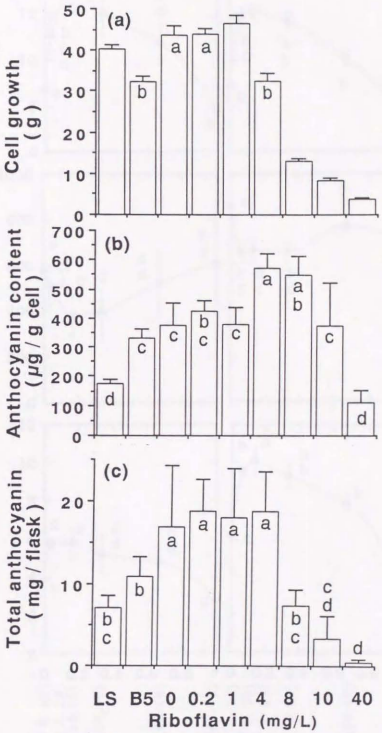


Fig.36 Effect of various riboflavin concentrations (0~40 mg/L) on cell growth and anthocyanin synthesis in suspension cultures of strawberry cells. Cultures were maintained in LS medium with modified ammonium and nitrate concentrations (2mM: 28mM), 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent the standard error of 3 replicates. a-d: Bars with same letter do not show significant difference at $P = 0.05$.

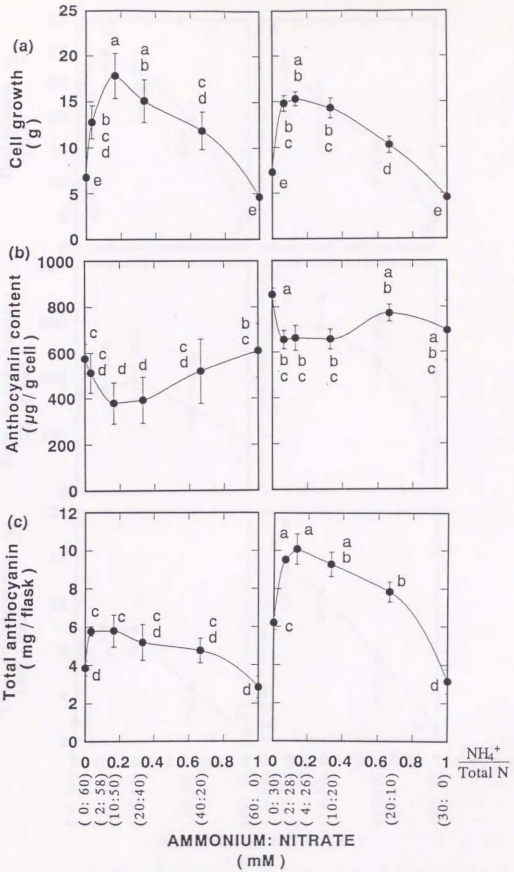


Fig. 37 Effects of riboflavin (4 mg/L) on cell growth and anthocyanin synthesis in various ratios of ammonium and nitrate using suspension cultures of strawberry cells. Cultures were maintained in LS medium with various ammonium and nitrate concentrations, 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 1 week. Vertical bars represent standard errors for six flasks. The symbols a-e mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

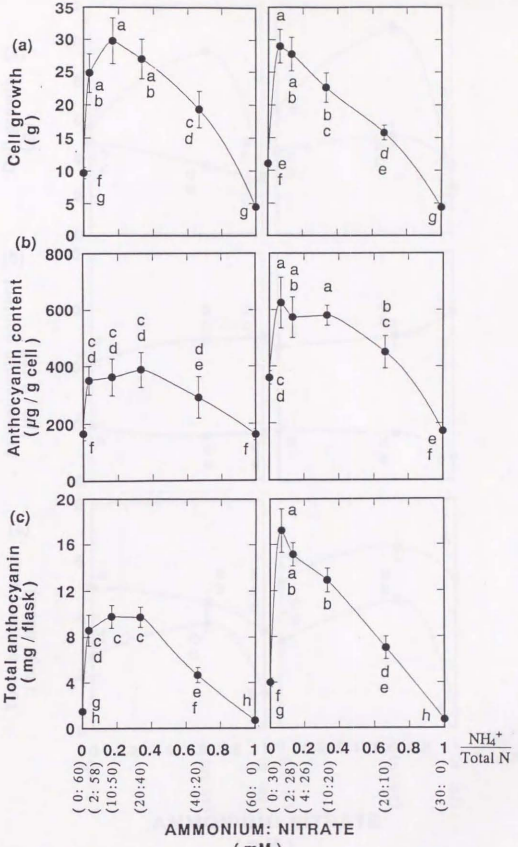


Fig. 38 Effects of riboflavin (4 mg/L) on cell growth and anthocyanin synthesis in various ratios of ammonium and nitrate using suspension cultures of strawberry cells. Cultures were maintained in LS medium with various ammonium and nitrate concentrations, 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent standard errors for six flasks. The symbols a-h mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

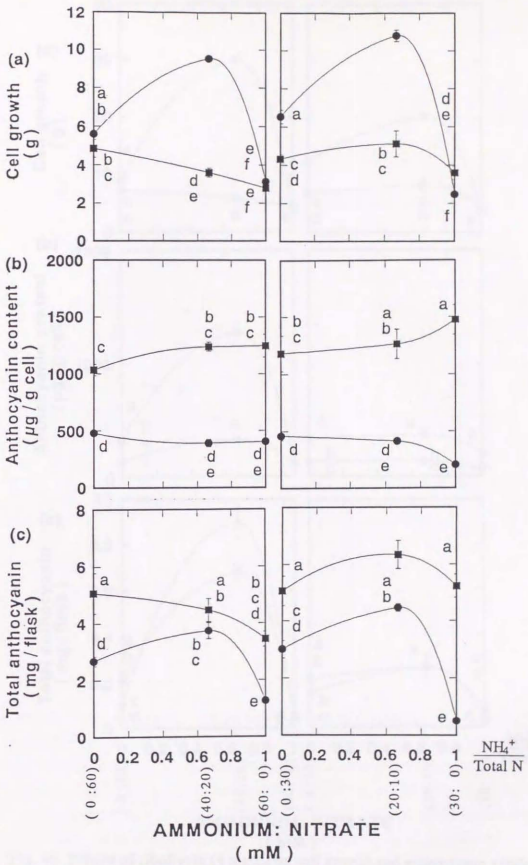


Fig. 39 Effects of riboflavin (4 mg/L) on cell growth and anthocyanin synthesis in various ratios of ammonium and nitrate using suspension cultures of strawberry cells. Cultures were maintained in LS medium with various ammonium and nitrate concentrations, 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 1 week. Riboflavin addition : ■, and control : ●. Vertical bars represent standard errors for three flasks. The symbols a-f mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

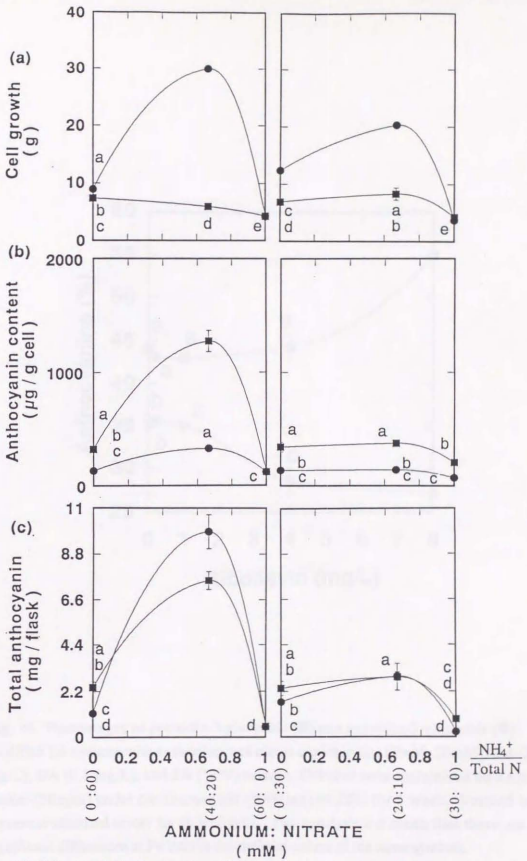


Fig. 40 Effects of riboflavin (4 mg/L) on cell growth and anthocyanin synthesis in various ratios of ammonium and nitrate using suspension cultures of strawberry cells. Cultures were maintained in LS medium with various ammonium and nitrate concentrations, 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Riboflavin addition : ■, and control : ●. Vertical bars represent standard errors for three flasks. The symbols a-e mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

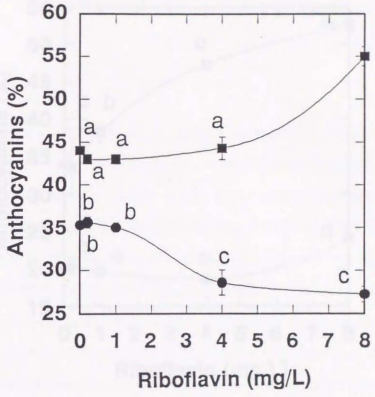


Fig. 41 Percentages of peonidin-3-glucoside (■) and cyanidin-3-glucoside (●) in modified LS medium with ammonium and nitrate concentration (2mM: 28mM), 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose. Cultures were maintained on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 1 week. Vertical bars represent standard errors for three flasks. The symbols a-c mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

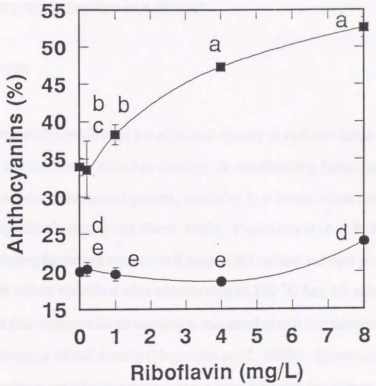


Fig. 42 Percentages of peonidin-3-glucoside (■) and cyanidin-3-glucoside (●) in modified LS medium with ammonium and nitrate concentration (2mM: 28mM), 2,4-D (1 mg/L), BA (0.1 mg/L), and 5% (W/V) sucrose. Cultures were maintained on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C for 2 weeks. Vertical bars represent standard errors for three flasks. The symbols a-e mean that there are no significant differences at P= 0.05 in the ordinate values of the same symbols.

CHAPTER 5

EFFECTS OF CONDITIONING FACTOR ON ANTHOCYANIN PRODUCTION IN STRAWBERRY SUSPENSION CULTURES

INTRODUCTION

Plant cells are usually cultured at a certain cell density in order to initiate cell division. This is called the minimum effective density. A conditioning factor exists in calli or cultured medium that induces cell growth, resulting in a lower value for the minimum effective density of cells (Stuart and Street, 1969). Yamakawa *et al.* (1985) also reported that the conditioning factor in a tobacco cell suspended culture reduced plating grape cell densities. This effect remained after autoclaving at 120 °C for 10 min. It has been generally noted that anthocyanin accumulation increased as cell inoculation size increased within a certain range of cell density (Nagarajan *et al.*, 1989). However, anthocyanin accumulation using a conditioning factor has not been reported for suspended cultures of plant cells. Furthermore, little work has been done on anthocyanin production using cultures of callus cells from strawberry plants suspended in liquid.

Therefore, in this study, the various effects of conditioned broth (filtrate of cultured medium) were examined concerning cell growth and anthocyanin accumulation of suspended strawberry cell cultures. The stimulation of anthocyanin accumulation and change in the major anthocyanin (cyanidin-3-glucoside and peonidin-3-glucoside) compositions in the cultured strawberry callus were observed after the addition of conditioned broth to the LS culture medium.

MATERIALS AND METHODS

Plant Materials and Callus Formation

Callus tissues were induced from leaves of *Fragaria ananassa* cv Shikinari. The leaves were obtained from aseptically regenerated plants and were placed on the LS medium containing 3% sucrose, 0.2% Gellangum (Wako Chemical), 0.1 mg/L of BA, and 1 mg/L of 2,4-D. Tissues were incubated at 25 ℃ under a 16 h-light, 8 h-dark cycle with light of 800 lux. Callus tissues were transferred every 3 weeks to a freshly prepared LS medium.

Cell Suspension Cultures

Cell suspension cultures were initiated by transferring about 2 g (fresh weight) of friable callus tissue to 100 mL of liquid LS medium supplemented with 3% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks. They were incubated on a rotary shaker (80 rpm) under continuous light of 800 lux at 25 ℃ for 3 weeks, during which the medium was changed every week. The inoculation rate was about 10% of precultured suspended cells. The resulting cell suspension (0.5~2 g fresh) was transferred to various freshly prepared test media (100 mL medium / 500 mL flask) and then incubated under continuous light of 8000 lux at 25 ℃ on a rotary shaker (80 rpm) to induce the production of anthocyanins.

Preparation of Conditioned Medium

The conditioned broth was prepared following way: culture broth was subcultured 3 times (1-week intervals) with about 2 g of cells in 100 mL of the fresh liquid LS medium containing 3% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks under continuous light of 800 lux, and the third sub-cultured medium was filtered using a nylon mesh (30 μm) and used as the conditioned medium.

Test of Conditioning Media

The effects of the conditioning phase duration on the activity of anthocyanin production were studied so as to obtain the optimal conditioned broth for strawberry cell suspension. The duration of the conditioning times (1~14 days) under continuous light

of 800 lux were varied, and then the addition of the filtrered conditioned broth (concentrations of 0, 25, and 100%) to the freshly prepared LS medium was changed. Cells were added to the prepared conditioned media and cultured under continuous light of 8000 lux. Then the stability of the conditioning factor to high temperatures was examined by boiling the medium for 10 min.

Test of Freeze-dried Conditioned Medium

To determine whether the effect of the conditioning factor on cell growth and anthocyanin accumulation was influenced by waste products from cells, the conditioned broth (500 mL) was freeze-dried, and added the dried medium (0.1~0.5 g) to the freshly prepared LS medium. Cell weights and anthocyanin contents were monitored.

Effects of Conditioned Medium on Anthocyanin Compositions

The volume of conditioned broth used was changed to study the effects of the conditioning factor on cyanidin-3-glucoside and peonidin-3-glucoside. Differences in composition of two major anthocyanin were also monitered by varying culturing time on conditioned media.

HPLC Analysis of Major Anthocyanins

Solution A (acetic acid : acetonitrile :water-20:25:55), diluted to 35% with water and containing 0.1% trifluoroacetic acid (TFA) at 4 ℃ was used to extract anthocyanins from fresh callus tissues of leaves to allow for HPLC analysis. The extract obtained was then diluted with 35% solution A to become a concentration of 1 OD (optical density) at 528 nm with a spectrophotometer. After filtration, 2 μL of the sample was injected and analyzed with HPLC (Waters 600E) using octadecyl silica (ODS) column (Develosil ODS-5 4.6 mm ϕ X 250 mm, Nomura Chemical), which was eluted with 35% solution A diluted with water containing 0.1% TFA at 40 ℃. The chromatograms were recorded with a Shimadzu C-R2A and calculated for the percentage of cyanidin-3-glucoside and peonidin-3-glucoside contents from the peak areas.

Determination of Anthocyanins and Cell Growth

Fresh callus tissue for anthocyanins were extracted overnight using a solution containing 0.1% HCl-MeOH at 4 ℃. After centrifugation at 1000 x g for 5 min, the absorbance of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated with the extinction coefficient ($E^{1\%}_{1cm}$=680 at 528 nm) which was obtained by using purified peonidin-3-glucoside from cultured strawberry cells as a standard. Total anthocyanin yield was expressed as mg/100 mL of liquid LS medium in a 500 mL cell culture flask. Cells were separated from the culture medium by filtration through a nylon filter (30 μm) and weighed. The results were expressed as fresh cell weight per flask.

Statistics

Data are presented as means ± SE (Standard error). Statistical analyses were made using the one-way analysis of variance (ANOVA). Differences of P (Probability) < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Best Conditioning Time

The effects of the conditioning factor on cell growth and anthocyanin production were investigated using strawberry cell suspension. Generally it is difficult to grow cells under low cell densities regardless of using agar culture or suspended culture. Muir *et al.* (1954) succeeded in establishing single cell cultures of marigold and tobacco, which were grown on filter paper using sunflower and tobacco calli as host cells. These results revealed the existence of a conditioning factor for growth promoting materials in callus cells. In strawberry cell suspensions, the amount of inoculum was an important factor in the time required to initiate the growth of suspended cultures. A small amount of inoculum (1 g / 100 mL) requires a long time for growth. Anthocyanin accumulation was also stagnated at inoculation levels less than 2 g / 100 mL (Fig. 8) suggesting that

the conditioning factor influenced anthocyanin accumulation in the same manner as seen for the cell growth.

Therefore, to determin the best conditioning time, cells (2 g fresh cells) were added to the conditioned broths and cell growth and anthocyanin content were monitored (Fig. 43). Cell growth diminished as the number of days of conditioning increased, and showed remarkable stagnation in the conditioned broth of five days to two weeks due to a lack of nutrients in the media. The cell growth pattern of a one-day conditioned broth was almost the same as that of the control broth. The cell weights for the cultures with conditioned broths of two-weeks and five-days were significantly higher after two days than that of control, indicating that the conditioning factor influenced cell growth during the early phase of cell culturing.

However, this phenomenon was not so clear as that shown in the experiment by Stuart and Street (1969). They reported dramatic cell growth (five-fold) of *Acer pseudoplatanus* using a conditioned broth that was conditioned for two days. Sakato and Misawa (1974) also reported the stimulation of cell growth (five-fold) by using a 50% concentration conditioned broth in combination with a new LS medium for *Camptotheca acuminata* cell suspension. The author has no explanation for this difference.

Anthocyanin accumulation was remarkably stimulated through the addition of a conditioned broth. Anthocyanin content was significantly higher ($P < 0.01$) after five days in the cultures using broths conditioned for 5- and 8-days. Here, the content reached about 2.4-fold that of the control broth (Fig. 43b). This effect was clearly seen in the early phase of culturing. It is clear that the enhancement of anthocyanin accumulation can not be attributed to the difference in cell growth (Fig. 43a), suggesting the presence of a conditioning factor, a substance that promotes anthocyanin accumulation in the conditioned broth. Therefore, it seems that about one week of conditioning is suitable to stimulate anthocyanin accumulation in strawberry cell suspension.

Since cultured broths were used as a conditioning media in this experiment, a drastic increase in total anthocyanin production was not observed. Broth conditioned for one

day yielded the highest anthocyanin production after 8 to 11 days. This result indicates that an increase in anthocyanin production as compared to the control is possible using a conditioning factor if there is a sufficient supply of nutrients in the culture medium (Fig. 43c).

Dose Response of Conditioned Medium

In order to investigate the dose response of the conditioned broth (after seven days of conditioning) on anthocyanin accumulation, media were prepared by adding various concentrations of the mixture to the new medium (Fig. 44). To clarify the effect of the conditioning factor on anthocyanin accumulation, an inoculum amount of 0.5 g (fresh cells) was used in this experiment. Higher cell growth is shown when using a 25% concentration of the conditioned medium as compared to the cell growth in the control broth after 8 days (Fig.44a). Anthocyanin accumulation was significantly enhanced when the broth that had been conditioned for 7 days was added to the medium, and ranked as 100%, 25%, and control in descending order. Anthocyanin accumulation in the medium with 100% concentration of the conditioning broth reached nearly eight-fold that of the accumulation in the control broth at day 5 (Fig. 44b). As shown in Fig. 44c, there is a suitable concentration of conditioned broth to increase anthocyanin production.

Heat Tolerance of Conditioning Factor

The effect of boiling the medium on the stability of the substance that promotes anthocyanin accumulation was also investigated (Fig. 45). The cell growth curve was almost the same as the that of the control broth. Moreover, no difference was observed in the anthocyanin content between the boiled broth and control broth, suggesting the substance that promotes anthocyanin accumulation in strawberry cells is non-volatile and thermostable.

Stuart and Street (1971) reported that the conditioning factor, as a growth promoting substance, in *Acer pseudoplatanus* was volatile. The difference between our results and

those of Stuart and Street (1971) may be related to the variations of plants, or suggest quite a different substance.

Effects of Freeze-dried Conditioned Broth

Furthermore, the possibilty of enhancing anthocyanin production using the conditioning factor was investigated. It is evident that anthocyanin production can be increased by adding a conditioned broth if the nutrient condition in the medium is adequate (Fig. 43 and 44). Hence the broth conditioned for seven days was tested for anthocyanin production after being freeze-dried. The time course and inoculum amount of the dried conditioned broth for anthocyanin production in suspended strawberry cells (Fig. 46) indicated that the inoculum amount influenced cell growth in the early phase of culturing (Fig. 46a), and also that the anthocyanin content was remarkably influenced by the amount of dried conditioned broth. The highest content (245 μg/g fresh cell) was obtained using 0.5 g addition of freezed-dried conditioned broth (Fig. 46b). Anthocyanin accumulation was significantly increased ($P< 0.05$) according to the inoculum amount on day four and six. Considering total anthocyanin production per flask (Fig. 46c), more than 0.5 g of inoculum may enhance the total anthocyanin yield in a 500 mL flask containing 100 mL of medium. Total anthocyanin reached the maximum of 9 mg / flask on day 12. This increase is not due to the sucrose present in the freezed-dried conditioned broth (Fig. 46b), and the activity of anthocyanin accumulation with glucose is half of the sucrose in mol unit (data not shown). Therefore, even if 0.5 g of the freez-dried materials is monosaccharides by complete assimilation of sucrose, it was impossible to enhance the anthocyanin accumulation like Fig. 46b by using only monosaccharides. Moreover, many researchers have reported that reduction of ammonium and phosphate promote the formation of anthocyanins (Do and Cormier, 1991; Yamakawa *et al.*, 1983b). However, the anthocyanin accumulation was remarkably stimulated by adding the freeze-dried conditioned broth to the freshly prepared LS medium, which indicates the enhancement of anthocyanin accumulation is caused by the conditioning factor for anthocyanin accumulating materials. Therefore, the

author thinks that anthocyanin production can be increased using the freeze dried conditioned broth in the strawberry suspension culture.

Effects of the Conditioning Factor on Anthocyanin Composition

The color of cultured strawberry cells changes according to the concentration of the conditioned broth added to the fresh culture medium, anthocyanin compositions were analyzed using HPLC (Fig. 47). The content of peonidin-3-glucoside fell significantly ($P< 0.01$) until the content of conditioned medium reached 75% of the total culture medium. When a 100% concentration of the conditioned broth was used, the content of peonidin-3-glucoside increased 70% of the total anthocyanin, while the content of cyanidin-3-glucoside decreased. The total of these two major anthocyanins equals about 85% of the total anthocyanins (in which every anthocyanin content was monitored), suggesting that the conditioning factor only influences the key enzymes that produce cyanidin and peonidin. This leads to the possibility of changing the anthocyanin composition by controlling the culturing time, due to the conditioning factor, i.e. the substance that promotes anthocyanin accumulation is released from the cells when cell density rises (Fig. 43b). Therefore changes in anthocyanin composition were monitored (Fig. 48). The unexpected results were obtained. The content of peonidin-3-glucoside increased, while that of cyanidin-3-glucoside decreased as the number of days of culture increased.

Stanko and Bardinskaya (1963) reported that, at the begining of formation of anthocyanins, the culture of *Parthenocissus tricuspidata* callus tissues contained mainly substances from the group of cyanidin-3,5-diglycosides, whereas at the end of growth, primarily methylated groups of delphindin-malvidin-3-monoglycoside and malvidin-3,5-diglucoside were present due to the start of necrosis.

This data agrees with the fact that methylation in anthocyanins increases as the number of days cultured proceeds. However it is difficult to explain the relationship between the conditioning factor on anthocyanins composition and methylation on anthocyanin as the number of days of culture increase. Of particular interest, in author's opinion, is to make

it possible to regulate anthocyanin composition and production using a conditioning factor. Therefore it is further necessary to investigate the role of the conditioning factor on the biosynthesis of anthocyanins.

CONCLUSION

1. Anthocyanin accumulation was greatly enhanced using media conditioned for one week.

2. Stimulation of anthocyanin synthesis using conditioned medium was clearly observed in relation to dose response.

3. The conditioning factor of the substance to promote anthocyanin synthesis was thermostable.

4. By adding freeze-dried conditioned broth to the culture medium, not only anthocyanin content but also total anthocyanin increased.

5. Conditioned medium influenced anthocyanin compositions. Both high-level (100%) and low-level (0%) of conditioned medium promoted formation of peonidin-3-glucoside at the expense of cyanidin-3-glucoside while intermediate levels had the reverse effect. However, for the case of the ordinary strawberry cell cultures, this phenomenon was not observed as the number of days of the culture growth increased.

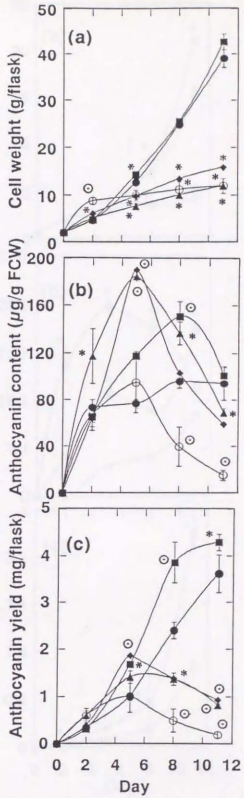


Fig. 43 The influence of the duration of the conditioning phase on cell growth and anthocyanin accumulation under continuous light of 8,000 lux in suspension cultures of strawberry cells. Cells (2g fresh weight) were cultured in various conditioned broths which were conditioning under continuous light (800 lux) with (1 mg/L) 2,4-D, (0.1 mg/L) BA, and 3% sucrose in LS medium on a rotary shaker (80 rpm) at 25°C. Duration of conditioning ●: Control 0 day; ■ : 1 day; ◆ : 5 days; ▲ :8 days; ○ : 2 weeks. Each value represents the averages of three replicates, vertical lines represent standard error of replicates. The symbols * and ⊙ mean that there are significant differences at P= 0.05 and 0.01, respectively, within the same day.

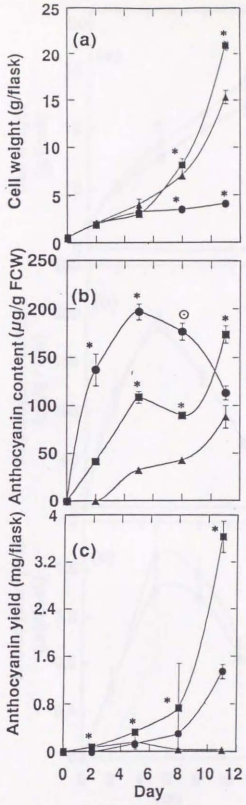


Fig. 44 The effect of conditioned medium concentration on cell growth and anthocyanin accumulation under continuous light of 8000 lux. Cells (0.5 g fresh weight) were cultured in various concentrations of conditioned broth, which was conditioned for seven days under continuous light (800 lux) with (1 mg/L) 2,4-D, (0.1 mg/L) BA, and 3% sucrose in LS medium on a rotary shaker (80 rpm) at 25°C. Concentration of the conditioned medium ●: 100% ; ■ : 25% ; ▲ : Control 0%. Each value represents the averages of three replicates, vertical lines represent standard error of replicates. The symbols * and ⊙ mean that there are significant differences at P= 0.05 and 0.01, respectively, within the same day.

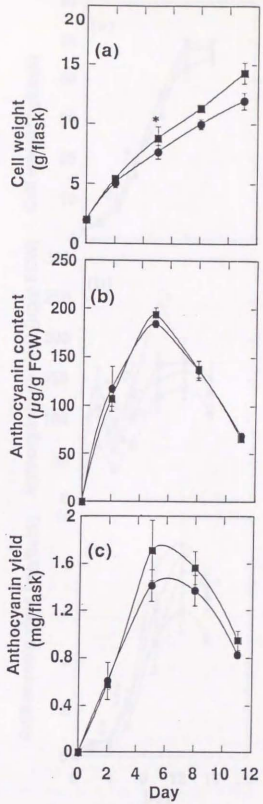


Fig. 45 The effects of boiling the conditioned medium on cell growth and anthocyanin production in suspension cultures of strawberry cells under continuous light of 8000 lux. Cells (2g fresh weight) were cultured in conditioned broth, which was conditioned for seven days under continuous light (800 lux) with (1 mg/L) 2,4-D, (0.1 mg/L) BA, and 3% sucrose in LS medium on a rotary shaker (80 rpm) at 25°C. Types of conditioned medium ●: Control ; ■ : Boiled for 10 min. Each value represents the averages of three replicates, vertical lines represent standard error of replicates. The symbol * means that there is significant differences at P= 0.05 within the same day.

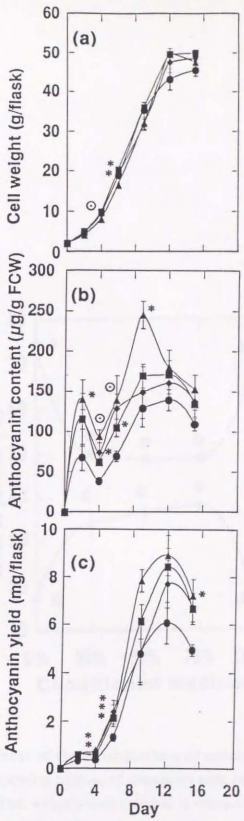


Fig. 46 The effects of freeze-dried conditioned broth on cell growth and anthocyanin production in suspension cultures of strawberry cells. Cells (2g fresh weight) were cultured in LS medium containing 2,4-D (1 mg/L), BA (0.1 mg/L), sucrose 3%, and various concentration of freeze-dried conditioned broth on a rotary shaker (80 rpm) under continuous light (8000 lux) at 25°C. Amount of freeze-dried 7 days conditioned broth ●: Control 0 g; ■ : 0.1g; ◆ : 0.3g; ▲ :0.5g. Each value represents the averages of three replicates, vertical lines represent standard error of replicates. The symbols * and ⊙ mean that there are significant differences at P= 0.05 and 0.01, respectively, within the same day.

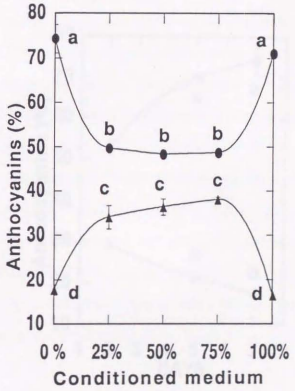


Fig. 47 The effects of the concentrations of conditioned broth on anthocyanin composition in suspension cultures of strawberry cells under continuous light of 8000 lux. Cells (0.5 g fresh weight) were cultured in various concentrations of conditioned broth, which was conditioned for seven days under continuous light (800 lux) with (1 mg/L) 2,4-D, (0.1 mg/L) BA, and 3% sucrose in LS medium on a rotary shaker (80 rpm) at 25°C, harvested and monitored for content of peonidin-3-glucoside (●) and cyanidin-3-glucoside (▲) at seven days. The symbols a-d mean that there are no significant differences at P= 0.01 in the ordinate values of the same symbols.

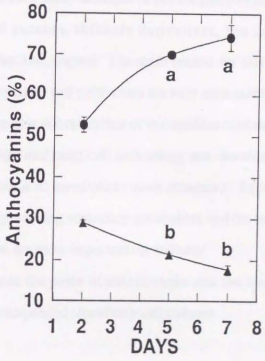


Fig. 48 The change in the percentage of peonidin-3-glucoside (●) and cyanidin-3-glucoside (▲) in LS medium. Cultures (0.5g fresh cells) were maintained with (1 mg/L) 2,4-D, (0.1 mg/L) BA, and 3% sucrose on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for seven days. The symbols a-b mean that there are no significant differences at P= 0.01 in the ordinate values of the same symbols.

CAPTER 6

PROCESS DESIGN OF ANTHOCYANIN PRODUCTION IN COMMERCIAL SCALE

INTRODUCTION

Although there have been many attempts to culture plant cells on a large-scale basis, only one commercial success, shikonin derivatives, has been achieved (Mitsui Petrochemical Industries. Ltd., Japan). The main reason for this lack of success is that metabolites produced by plant cell cultivation are very expensive due to the long mean generation times and the low concentration of metabolites contained in cells. Advances in both plant-cell biology and plant-cell technology are therefore required in order to make industrial production of metabolites more attractive. Especially, balance of the economical aspects for producing secondary metabolites and the technological limitations of mass-scale cultivation are most important for industry.

This chapter investigates the price of anthocyanin and the cost to produce it at the commercial scale using suspended strawberry cell cultures.

RESULTS AND DISCUSSION

Cost for anthocyanin production

Generally the personnel expenditures of a large corporation reach approximately 3,000-4,000 yen per hour for one laborer. This price fluctuates according to the business and throughout the year. In this chapter, the estimation of labor cost was carried out applying a personnel expenditure assumed at 4,000 yen per hour for one laborer. Therefore, the personnel expenditure for one month (it is assumed eight hours per day and 20 days per month) is 640,000 yen. If 1.8 KVA is applied as the electricity consumption for shaking, light and heater to produce anthocyanin, and the system is

assumed to work continuously for one month, the total electricity consumption is 1,296 KWh, and the charge would be 21,157 yen (18,817 yen :14.52 yen/ KWh + 2,340 yen :basic charge). Therefore, combined personnel expenditure and electric charge is 661,157 yen/ month. The medium charge was also calculated based on WAKO chemical catalog. The cost of LS medium containing 1 mg/L 2,4-D, 0.1 mg/L BA and 3% sucrose (V/W) was approximately 61 yen/L. However, this cost is far cheaper and incomparable with the personnel expenditure and electricity charge, therefore the personnel expenditure was regarded as the main cost for large-scale strawberry cell culturing.

On the other hand, anthocyanins have generally been sold for 10,000 yen (λmax of the solution OD 50/L). This indicates that about 730 mg of anthocyanin is contained in one liter if the estimation of anthocyanin content is calculated based on $E^{1\%}_{1cm}$=680 at 528 nm of peonidin-3-glucoside which was produced as the main anthocyanin from suspended strawberry cell. This content equals approximately 4900 mL produced in modified LS medium containing 5% sucrose (Fig. 32). The cost of the medium is approximately 299 yen. Therefore, real returns of the 4900 mL culture was calculated as 9,701 yen. Then, to further cover the personnel expenditure and electricity charge in one month (661,157 yen), about 334 L of cell culture is needed (4.9 × 661,157/ 9,701 = 334 L). Here, the cost for anthocyanin production is calculated based on only the personnel expenditure and the charge of total electricity consumption. However, the cost of equipment and facilities for anthocyanin production and purification must also be added to the cost. Therefore, suspended strawberry cell cultures must produce more than 334 L of anthocyanin per month. However, the cost of equipment and facilities for anthocyanin production and purification must also be added to the cost. It is further necessary to give consideration to the cost of depreciation for the equipment and facilities, the cost of repair and taxation for the facilities. Therefore, the calculation in this chapter given as a zero-order approximation.

Large-scale cultivation of plant cells at high densities can be considered an effective method of accomplishing industrial production of useful metabolites in suspended cultures. The two main technological problems to be overcome are : (1) the difficulty of oxygen supply to plant cells, and (2) the adverse effects of hydrodynamic stress generated by the aeration-agitation operations usually used in supplying oxygen to plant cells.

Hong *et al.* (1989b) reported on the growth kinetics of suspended strawberry cell cultures in shake flask, airlift, stirred-jar and roller bottle bioreactors using cells from the Brighton strawberry. Although differences in the volumetric oxygen-transfer coefficient were not observed in the relationships between anthocyanin production and these cultivation systems, the specific oxygen consumption rates of the suspended strawberry cultures in shake flasks ranged from 0.10 to 0.17 mmol O_2/gDCW/hr. They showed these values were relatively low compared to that of specific oxygen consumpition rates in other plant tissue culture systems. However, the value of oxygen-transfer coefficient in the shake flask was higher than any other cultivation system. Furthermore it was demonstrated that the specific growth rates of suspended strawberry cell cultures were higher in roller bottles equipped with baffles (0.15 /day) than any other system. Therefore, this data provides a solution to the difficulty of supplying oxygen adequately to suspended strawberry cells.

The Brighton callus is only capable of producing anthocyanin when cultured under light irradiation (Hong *et al.*, 1989a), and this fact conforms completely with production of Shikinari callus as well. Therefore, based on this data, if the culture medium is suitable for anthocyanin synthesis, regulation of light intensity is the most important factor for anthocyanin production when culturing cells in roller bottles, and this may be applied to the production of Shikinari callus.

Tanaka (1987) has succeeded in the large-scale production of shikonin derivatives from *Lithospermum erythrorhizon* cells in 1000 L culture. He also examined the yield of shikonin using five different types of fermentor. It was revealed that a rotating-drum

fermentor was best to increase cell culturing without decreasing the relative yield of shikonin produced using shaking flasks.

Concerning the shape of bioreactors, both researchers agreed that the roller-bottle type was best for large-scale cell culturing. These two examples did not apply light irradiation because the purpose was only to measure the cell growth of strawberry cultures and light has an inhibitory effect on the accumulation of shikonin; however, these results are very useful to solve the hydrodynamic stress generated by the aeration-agitation operations.

Zhong *et al.* (1991) demonstrated anthocyanin production at the bioreactor level (5L) using suspended *Perilla frutescens* cells under light irradiation. They succeeded in enhancing the plant cell culturing process from a shake flask to an air-sparged system without reducing productivity. The maximum anthocyanin production obtained was 2.9 g/L using an aerated and agitated bioreactor (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with light irradiation at 27.2 W/m^2.

These results further suggest the possibility of realizing large-scale anthocyanin production using suspended *Fragaria ananassa* cv Shikinari cells, because it is easy to attach lighting facilities for cell culturing to this type of roller bottle bioreactor. The difficulty of increasing cell culturing using Shikinari cells only is attributed to the necessity of light irradiation for anthocyanin formation. On the other hand, Kobayashi *et al.* (1993) reported anthocyanin production in a 500 L pilot bioreactor without light irradiation using *Aralia cordata* cells. By administering CO_2 into the culturing vessel, the yield was 62.2 Kg of fresh cells with 545 g anthocyanin content after 16 days. From this result, an anthocyanin-producing cell that does not require light irradiation can be obtained by screening strawberry cells. Therefore, it might be possible to use cultures of more than 500 L at a time.

Comparison of anthocyanin production with intact flower

Tremendous labor will be saved if it is possible to extract anthocyanin from intact plants or flowers rather than producing it using plants cell cultures because it does not require large equipment and facilities for cell culturing. Therefore, it is better to extract

anthocyanin from intact turnips or flowers if the purpose is only to obtain anthocyanin. For instance, about 1.5 g of anthocyanin was extracted from intact flowers (150 g,fresh weight) of the *Dahlia pinnata* Cav (Hayashi, 1933). This amount of anthocyanin is 100 times greater than that produced by using suspended strawberry cells in 100 mL medium. Only 39 mg of anthocyanin can be produced using suspended strawberry cells in a flask per year. This suggests that the total anthocyanin production using suspended strawberry cells in a flask cultured for one year is far less than that of the intact flower of *Dahlia pinnata* Cav. The number of harvests of the suspended strawberry cells is only 26 for one year if it is assumed that the cell culture duration is two weeks (Fig. 32).

Approximately 900 g ($0.015g \times 500L / 0.1L \times 12$) of anthocyanin can be produced when suspended strawberry cells are cultured by the 500 L/ month scale for one year. This amount of anthocyanin is the same as that produced by 600 dahlia plants if we assume that 150 g of flowers is obtained from one plant. The 600 dahlias can be grown in a field approximately 150 m^2 in area.

CONCLUSION

1. To cover the production cost, at least 50 g of anthocyanin must be produced from suspended strawberry cell cultures. This means approximately 334 L of cell culture must be used over a period of one month in a year.

2. Sufficient oxygen supply and reduced hydrodynamic stress, which is generated by the aeration-agitation operations, can be realized using a roller-bottle bioreactor. If the scale of culturing is not limited by light irradiation, it is possible to increase the bioreactor size to at least a 500 L jar fermentor by further modifying culturing conditions.

CHAPTER 7

CONCLUSION

The objectives of this study are to establish a useful technology to produce anthocyanins using strawberry suspended cells and further to examine the various factors influencing anthocyanin production. Conclusions of this study are given below.

In Chapter 1, conditions for producing white friable callus and accumulating anthocyanin on the surface of the calli were studied using strawberry, cv shikinari, tissue. The white friable callus was obtained from three different parts of the plant (leaf, apical meristem and petiole) only when the medium concentrations were 2,4-D 0.5 mg/L or higher and BA below 2 mg/L, and light irradiation was 800 lux. Anthocyanin accumulated on the surface of the white friable callus when cultured under 3000 lux; however, the cells which the anthocyanin accumulated did not proliferate. Therefore, it was deduced that to produce anthocyanin using cultured strawberry cells, the cells must first be cultured under a dim lighting (800 lux) to enhance cell growth, and then irradiated at an intensity of more than 3000 lux.

In Chapter 2, studies to stably produce anthocyanin in suspended strawberry cultures were carried out, and the major anthocyanins produced in cultured cell were identified. The results indicated that to produce anthocyanin in suspended, sub-suspended cultures from a solid culture medium under dim lighting (800 lux) was needed at least three times (1-week intervals). It was elucidated that cell inoculum size is an important factor, and 2 g of cell inoculum using leaf callus is best to produce anthocyanin in 500 mL flasks containing 100 mL medium after culturing for two weeks. Concerning anthocyanins, about eight kinds of anthocyanins were detected, and the two major anthocyanins from cultured strawberry cell were identified as peonidin-3-glucoside and cyanidin-3-glucoside.

In Chapter 3, various culture conditions were investigated to increase anthocyanin productivity in suspended strawberry cell cultures. The results revealed that not only

total anthocyanin production but also anthocyanin compositions were greatly influenced by various factors such as phytohormones, basic culture media, types of sugar, sucrose concentrations, total nitrogen and the ratio of ammonium and nitrate. The total anthocyanin was produced in a modified LS medium containing ammonium (2 mM) and nitrate (28 mM) with 5% sucrose reached 15 mg/100 mL, and this was the highest in the modified basic culture conditions.

Furthermore, to increase anthocyanin production, the effects of riboflavin on anthocyanin synthesis and cell growth were investigated in Chapter 4. The results indicated that riboflavin was very effective to increase anthocyanin synthesis, and even reduced the culture duration required for obtaining maximum total anthocyanin when petiole, apical meristem or leaf callus, which reduced the potential of anthocyanin formation, were cultured in medium containing riboflavin. The effect of riboflavin on increasing anthocyanin content was indeed obvious according to the low concentration of applied riboflavin even when leaf callus, which has the best potential to produce anthocyanin, was used. However, for total anthocyanin production, a positive effect from riboflavin was not observed because it inhibited cell growth.

In Chapter 5, the effects of conditioning factors on anthocyanin production were investigated. Although conditioning factors have been known to stimulate cell growth, this is the first report to show that conditioning factors also stimulate anthocyanin synthesis. Anthocyanin synthesis increased when cells were cultured in filtrated medium conditioned for one week, and a dosage response was observed in the anthocyanin content with the addition of conditioned medium. Total anthocyanin production also increased when freeze-dried conditioned medium supplemented freshly prepared LS medium. While conditioning factors promoted anthocyanin synthesis, they were also shown to have an influence on anthocyanin compositions. These results provide not only a new factor to promote anthocyanin production but also a new aspect to develop plant physiology concerning anthocyanin synthesis.

The assessment of the process design of anthocyanin production was investigated in Chapter 6.

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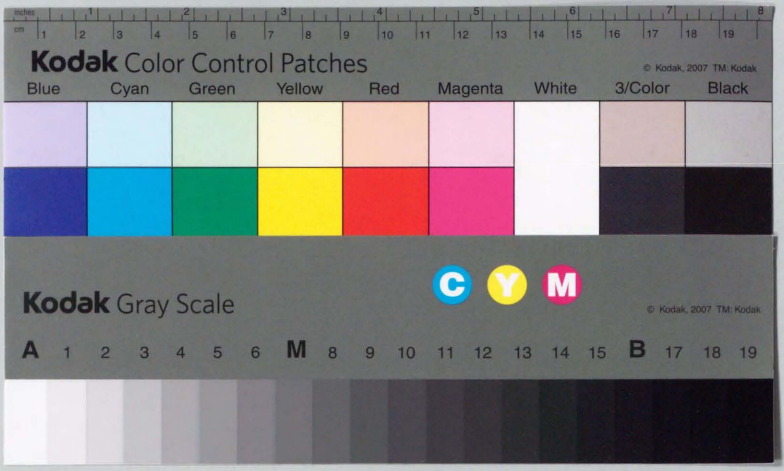
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