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Effect of Ammonium Ion and Temperature on Anthocyanin Composition in Sweet Potato Cell Suspension Culture

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Abstract

Ammonium level in culture medium and culture temperature was identified as having a significant influence on the anthocyanin composition of cell suspension culture originating from the storage root of sweet potato (*Ipomoea batatas* L.), cv. Ayamurasaki. In the presence of 20 mM NH_4^+ in the culture medium the dominant pigment was YGM-0a, a non-acylated anthocyanin. With the decrease of ammonium concentration a large increase of YGM-0f', an acylated anthocyanin, occurred. It was concluded that high ammonium concentration inhibits acylation of YGM-0a. The increase of culture temperature to 30 ℃ resulted in accumulation of pigments, which appeared on the ODS-column HPLC with earlier retention time, with the predominance of YGM-0a. At lower temperatures of 15, 20 and 25 ℃ the dominant pigment was YGM-0f'. It was concluded therefore that high temperature also inhibits acylation of YGM-0a.

Introduction

Plant cell cultures are becoming an important biotechnological technique to produce secondary metabolites, such as shikonin (Shimomura *et al.*, 1991) and ginsenosides (Yoshikawa *et al.*, 1997) on mass scale. Recently in the food industry there is a growing demand for use of natural food colorants. Purple-fleshed sweet potato has been regarded as a good source of stable anthocyanins, red food colouring pigments (Odake *et al.*, 1994) with light and heat stability equal to those of red cabbage and superior shelf life (Odake, 1997). A high anthocyanin producing cell line (PL) has been established from the storage root of purple-fleshed sweet potato (*Ipomoea batatas* L.), cultivar "Ayamurasaki" (Konczak-Islam *et al.*, 2000). However, the quality of major pigments accumulated in the culture was different from those produced by field grown storage root. While major pigments accumulated in storage root are highly acylated cyanidins and peonidins, in cell culture non-acylated cyanidin 3-sophoroside-5-glucoside dominated. Further research was therefore undertaken to identify factors, which could contribute to the accumulation of acylated pigments in PL suspension culture, similar to those produced in intact sweet potato storage roots.

Reports on the influence of different culture conditions on the quality of pigments accumulated *in vitro* are limited. In the suspension culture of grape (*Vitis vinifera* L.) high sucrose and low nitrate levels enhanced the intracellular accumulation of peonidin 3-glucoside (Do and Cormier, 1991a). In the same culture an increase of ammonium concentration from 2 to 8-16 mM decreased accumulation of peonidin 3-glucoside and instead peonidin 3-p-coumaroylglucoside was produced (Do and Cormier, 1991b). It was concluded that high ammonium concentrations could promote the acylation of peonidin 3-glucoside. In strawberry suspension culture with an increase in the NH_4^+: NO_3^- ratio at a nitrogen concentration of 30 mM, peonidin 3-glucoside increased and cyanidin 3-glucoside decreased. A similar effect was observed at 60 mM total nitrogen except when the ratio was 60:0, in which case the contents of both cyanidin 3-glucoside and peonidin 3-glucoside were directly opposite to the result at 30 mM (Mori and Sakurai,

1994). Clearly more detailed research is needed to identify the effect of both ions on the anthocyanin biosynthesis in cell suspension cultures.

The effect of temperature on cell growth and on the amount of secondary metabolites accumulated *in vitro* has also been studied. A low temperature of 22 ℃ reduced cell growth and anthocyanin accumulation in carrot suspension culture (Dougall *et al.*, 1983). In *Perilla frutescens* suspension culture anthocyanin production was reduced and cell growth increased at the relatively high temperature of 28 ℃ , and 25 ℃ appeared to be optimal for maximum pigment productivity (Zhong and Yoshida, 1993). In cell cultures of *Catharanthus roseus* the profile of accumulated alkaloids changed with the temperature regime. Maximum serpentine yields reached a peak between 20 and 25 ℃ , while maximum ajmalicine accumulation occurred at 20 ℃ (Morris, 1986). For the maximum alkaloid productivity in this culture the author proposed "stepped temperature profile during the culture period": the cells to be grown at 30 ℃ for 2 days followed by 22 ℃ for 12 days. The same approach appeared to be successful in increasing the amount of anthocyanin in the cell culture of strawberry (Zhang *et al.*, 1997). To our knowledge, temperature effect on pigment quality was not reported.

Knowledge of factors, which affect composition of *in vitro* accumulated pigments would be of advantage for commercial production of natural food colorants using tissue culture techniques. Therefore, the main objective of this study was to investigate the effect of selected medium components and environmental factors on the quality of pigment accumulated in PL suspension culture.

Materials and Methods

Callus culture

The callus was developed from the sweet potato storage root, cv. Ayamurasaki, as described previously (Konczak-Islam *et al.*, 2000). Suspended cell cultures were initiated by transferring about 1g (fresh weight) of callus to 25 m*l* of liquid medium in 100-m*l* Erlenmayer flasks. To obtain a fresh weight the aggregates were removed from medium and washed with 3% sucrose solution. Subsequently, they were separated from the liquid by filtration under vacuum and weighed. Basal Murashige and Skoog (MS, Murashige and Skoog, 1962) medium supplemented with 2 mg l^{-1} 2,4-D was used as a multiplication medium (MM). The cultures were incubated on a rotary shaker (130 rpm) at 25 ℃ in the dark. The medium was changed weekly.

The cells from suspension cultures, maintained for at least 3 months, were used for the experiments. From seven-day-old subcultures, 100 mg of cell aggregates were placed in 50-m*l* Erlenmayer flasks containing 10 m*l* medium of each treatment (pH 5.8 before autoclaving). All experiments were conducted with at least 4 replicates. The samples were collected after 14 days of culture unless otherwise stated.

Effect of nitrogen sources

Experiment 1. The influence of NH_4NO_3 on cell growth and anthocyanin accumulation was investigated through modifying the basal MS medium to contain 0, 2.5, 5, 7.5, 10, 15 and 20 mM of NH_4NO_3. The other N source of basal MS medium was 18.8 mM KNO_3. The media contained 3% sucrose. Growth regulators were omitted.

Experiment 2. Basal MS medium was modified to contain 4.7, 9.4, 18.8, 37.6, or 56.4 mM KNO_3. Concentration of NH_4NO_3 was kept at a level of 2.5 mM. Consequently, the following levels of NO_3^- were achieved: 7.2, 11.9, 21.3, 40.1 and 58.9 mM. As in the 7.2 and 11.9 mM NO_3^- treatments the level of K^+ ion became lower than in MS basal medium, these treatments were repeated with addition of KCl in the media to adjust the K^+ levels with that of MS basal medium (14.1 and 9.4 mM KCl, respectively). In all the media treatments 3% sucrose was present. Growth regulators were omitted. A basal MS medium enriched with 3% sucrose was used as a control.

Temperature effects

In temperature effect studies, modified MS medium with 2.5 mM NH_4NO_3 and 9.4 mM KNO_3, without growth regulators and with sucrose level elevated to 5% was used. The cultures were placed in growth chambers with constant temperature of: 15 ℃ , 20 ℃ , 25 ℃ and 30 ℃ . The samples were collected after 21 days.

Determination of growth

Growth was measured by removing the aggregates from the medium, washing them with a 3% sucrose solution, separating them from the liquid by vacuum filtration and weighing them. The growth index was defined as W/W_0, where W_0 and W denote fresh weight of the aggregates before and after the cultivation, respectively.

Extraction of anthocyanin

Cell aggregates separated from the culture medium by vacuum filtration were ground and steeped in 50 % acetic acid for 1 h. The volume of acetic acid solution was adjusted to 20 times equivalent of the sample weight. The samples were centrifuged at

10000 rpm for 10 min. The supernatants were used for anthocyanin identification and quality analysis.

Anthocyanin identification and HPLC analysis

The supernatant diluted fourfold with McIlvaine's buffer solution (Hodgman, 1954), pH adjusted to 3.0, was used for the measurement of the optical densities at 530 nm with spectrophotometer CS-9300PC (Shimadzu, Japan). Colour value (CV) of the pigment extract was calculated using the following formula: $CV=0.1xOD_{530}x4x20$ (CV/g tissue fresh weight), where OD_{530} is the spectrophotometric reading at 530 nm, and 4 and 20 are the levels of dilution (Shimizu and Nakamura, 1993).

HPLC analysis was performed according to the method described by Odake *et al.* (1992) on an LC-9A (Shimadzu, Japan) liquid chromatograph. Analytical HPLC was run on an Inertsil ODS-2 column (250 × 4.6mm, GL Sciences Inc.) at 35 ℃ and monitored at 530 nm. The following solvents in water with a flow rate of 1 m*l* min^{-1} were used: A - 1.5% phosphoric acid and B - 1.5% phosphoric acid, 20% acetic acid and 25% acetonitrile. The elution profile was a linear gradient elution for B of 25% to 85% during 40 min in solvent A. The chromatograms were recorded and the relative concentration of pigments was calculated from the peak areas. Identification of anthocyanins was carried out comparing the peaks with standard peaks of PL cell line and purple-fleshed sweet potato YGM anthocyanins: YGM-0a [cyanidin 3-*O*-sophoroside-5-*O*-glucoside], YGM-0f' [cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-*O*-β-*D*-glucopyranoside], YGM-1a [cyanidin 3-(6,6'-caffeyl*p*-hydroxybenzoylsophoroside)-5-glucoside], YGM-1b [cyanidin 3-(6,6'-dicaffeylsophoroside)-5-glucoside], YGM-2 [cyanidin 3-(6-caffeylsophoroside)-5-glucoside], YGM-3 [cyanidin 3-(6,6'-caffeylferulylsophoroside)-5-glucoside], YGM-4b [peonidin 3-(6,6'-dicaffeylsophoroside)-5-glucoside], YGM-5a [peonidin 3-(6,6'-caffeyl*p*-hydroxybenzoylsophoroside)-5-glucoside], YGM-5b [cyanidin 3-(6-caffeylsophoroside)-5-glucoside] and YGM-6 [peonidin 3-(6,6'-caffeylferulylsophoroside)-5-glucoside] according to Odake *et al.* (1992) and Terahara *et al.* (1999, 2000).

Results and Discussion

Effects of ammonium nitrate and potassium nitrate on pigment composition

Drastic changes in pigment composition were detected among the PL suspension cultures grown under different concentrations of NH_4NO_3 in culture medium. At high concentration of NH_4NO_3 dominated pigments, which appeared on ODS-column HPLC with early retention times, such as YGM-0a, -0a' and -0b and with the decrease of NH_4NO_3 level dominated pigments with later retention time, such as YGM-0f', -0g', -7a and -7e (**Fig. 1**). At 20 mM NH_4NO_3 the pigments with early retention times (YGM-0a, -0a', and -0b) comprised 48.7% of total anthocyanins as calculated by peak area (**Table 1**). The same pattern was observed at 15, 10 and 7.5 mM NH_4NO_3, where these three anthocyanins made up 50.4%, 48.5% and 49.8%, respectively. However, with the decrease of NH_4NO_3 below 7.5 mM the relative concentrations of these anthocyanins drastically decreased and reached the level of 34.0% at 5mM NH_4NO_3, 8.7% at 2.5mM NH_4NO_3 and 2.3 % when NH_4NO_3 was omitted. The decrease in relative concentrations of the early peaks was concomitant with an increase in the relative concentrations of YGM-0f', -0g', -7a and -7e peaks which appeared with later retention times. In the medium without NH_4NO_3 the concentration of these four pigments reached 66.1 % and was 3 times higher than their concentrations in the presence of 7.5 - 20 mM NH_4NO_3. With the reduction of NH_4NO_3 level from 7.5 to 0 mM a drastic increase in the relative concentration of YGM-7a

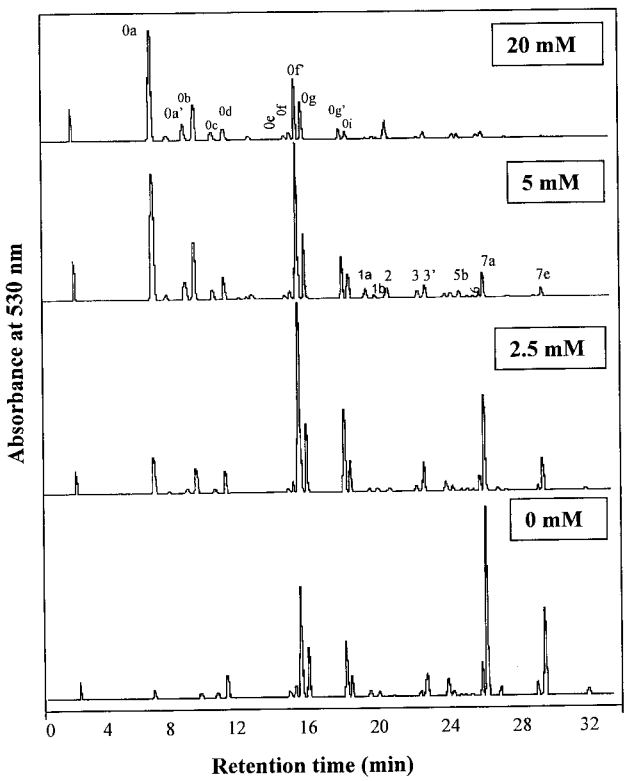

Fig. 1 HPLC chromatogram of the PL suspension culture at different level of NH_4NO_3 in the medium. The characters in the chromatogram are the YGM numbers of sweet potato anthocyanins according to Odake *et al.* (1992) and Terahara *et al.* (1999, 2000).

Table 1. Anthocyanin composition of PL suspension culture induced from storage root of *Ipomoea batatas*, cv. Ayamurasaki, after 14 days of culture at different level of NH_4NO_3 in MS medium. The values in the table are percentage of total anthocyanin calculated from the peak area at 530nm

Peak	NH_4NO_3(mM)						
	0	2.5	5	7.5	10	15	20
YGM0a	1.5 ± 0.1*	4.5 ± 1.7	19.0 ± 29	27.2 ± 1.8	27.9 ± 1.5	28.1 ± 2.5	29.3 ± 1.1
YGM0a'	ND	1.2 ± 0.7	5.3 ± 1.2	9.3 ± 0.9	7.9 ± 0.4	9.2 ± 2.8	7.8 ± 0.6
YGM0b	0.8 ± 0.4	3 ± 1.1	9.7 ± 1.2	13.3 ± 1.5	12.7 ± 0.9	13.1 ± 3.0	11.6 ± 1.0
YGM0c	0.5 ± 0.4	0.7 ± 0.2	1.3 ± 0.2	2.0 ± 0.4	1.9 ± 0.3	1.8 ± 0.1	1.7 ± 0.2
YGM0d	2.9 ± 0.8	3.8 ± 0.1	3.9 ± 1.1	4.1 ± 0.5	3.8 ± 0.4	3.8 ± 0.7	3.8 ± 0.4
YGM0e	0.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
YGM0f	1.5 ± 0.3	2.3 ± 0.1					
YGM0f'	17.8 ± 1.7	28.2 ± 2.8	27.8 ± 4.2[a]	20.5 ± 3.5[a]	17.1 ± 1.0[a]	14.7 ± 2.0[a]	15.1 ± 1.4[a]
YGM0g	6.3 ± 0.8	5.5 ± 0.7	6.1 ± 0.5	5.5 ± 0.5	6.4 ± 0.08	7.2 ± 2.2	7.4 ± 0.5
YGM0g'	8.8 ± 2.5	10.2 ± 0.9	7.0 ± 1.5	4.6 ± 1.2	3.1 ± 0.3	2.6 ± 0.04	2.5 ± 0.3
YGM0i	3.0 ± 0.7	2.8 ± 0.3	2.0 ± 0.4	1.7 ± 0.1	1.9 ± 0.4	2.1 ± 0.2	2.0 ± 0.2
YGM1a&b	0.7 ± 0.4	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.3	0.6 ± 0.3
YGM2	0.4 ± 0.3	0.6 ± 0.1	1.4 ± 0.5	2.5 ± 1.1	2.8 ± 0.9	3.4 ± 0.9	4.8 ± 0.1
YGM3&3'	5.2 ± 1.3	4.4 ± 0.8	2.1 ± 0.4	1.6 ± 0.4	1.9 ± 0.2	2.1 ± 0.9	2.6 ± 0.2
YGM5a&b	3.9 ± 0.6	2.9 ± 0.5	1.5 ± 0.1	1.8 ± 1.1	1.6 ± 0.3	1.6 ± 0.4	1.9 ± 1.0
YGM6			0.4 ± 0.2	0.6 ± 0.1	0.6 ± 0.3	0.8 ± 0.3	0.8 ± 0.1
YGM7a	25.9 ± 2.1[b]	16.2 ± 4.0[b]	4.6 ± 2.3	1.5 ± 0.3	1.3 ± 0.5	0.9 ± 0.2	1.0 ± 0.3
YGM7e	13.6 ± 3.8	5.5 ± 0.9	1.1 ± 0.6	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1

* Standard deviation of 3 independent determinations.

[a] Including minor peak YGM0f, [b] Including minor peak YGM6, ND: not detected.

was observed: from 1.5 to 25.9%, respectively. This increase was directly opposite to the decrease of YGM-0a level. The data indicates that the decrease of NH_4NO_3 level from 7.5 to 0 mM promotes accumulation of YGM-7a in PL suspension culture.

Pigments, which appear on ODS-column HPLC with early retention time are highly hydrophilic and have a simpler molecular structure than pigments, which appear with later retention times. Terahara *et al.* (2000) have identified the peak YGM-0a as a cyanidin 3-O-sophoroside 5-O-glucoside, a non-acylated anthocyanin, and the peak YGM-0f'as a cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-O-β-D-glucopyranoside, the first anthocyanin acylated with *p*-coumaric acid identified among sweet potato pigments. The high relative concentration of non-acylated YGM-0a at high NH_4NO_3 levels and its drastic decrease at lower NH_4NO_3 levels, concomitant with a drastic increase of acylated pigments, such as YGM-0f' clearly suggests that NH_4NO_3 in amount higher than 7.5 mM inhibits acylation of cyanidin 3-sophoroside-5-glucoside.

The changes observed in the pigment quality of PL suspension culture might be due to the decrease of NH_4^+ and/or NO_3^- level. Therefore, the effect of different NO_3^- levels was examined by changing the amount of KNO_3 in the medium while maintaining a constant level of 2.5 mM NH_4NO_3. The composition of pigments in cells grown under different NO_3^- levels in the medium appeared stable (**Table 2**). At all NO_3^- concentrations the major pigments accumulated were YGM-0f', followed by YGM-0g' and -7a. These three pigments contributed about 50 % of the total anthocyanin and were identical to those in the treatment with 2.5 mM NH_4NO_3 in the previous experiment. This result indicates that the composition of pigments accumulated in PL suspension culture is influenced by the concentration of ammonium ion in the medium. Therefore, with the selection of ammonium level in a production medium we might be able to regulate the quality of major pigments produced by PL suspension culture.

Effects of ammonium nitrate and potassium nitrate on the amount of accumulated pigment and cell growth.

High levels of nitrogen sources in culture medium inhibited anthocyanin accumulation in PL suspension culture. The reduction of NH_4NO_3 level from 10 to 2.5 mM in the medium resulted in an increase of total anthocyanin accumulation. The colour value

Table 2. Anthocyanin composition of PL suspension culture induced from storage root of *Ipomoea batatas*, cv. Ayamurasaki after 14 days of culture at different level of KNO_3 in modified MS medium. The values in the table are percentage of total anthocyanin calculated from the peak area at 530nm

Peak	NO_3^-(mM)							MS
	7.2	7.2*	11.9	11.9*	21.3	40.1	58.9	
YGM0a	6.6 ± 1.5[a]	3.95 ± 1.1	5.1 ± 0.8	6.4 ± 1.9	6.0 ± 1.7	3.8 ± 0.5	3.4 ± 0.5	26.7 ± 0.7
YGM0a'	2.5 ± 0.2	2.3 ± 0.2	T	T	T	ND	ND	6.1 ± 0.5
YGM0b	4.8 ± 0.3	2.8 ± 1.7	2.6 ± 0.4	4.8 ± 2.6	3.2 ± 1.3	2.3 ± 0.9	1.7 ± 0.3	15.7 ± 1.4
YGM0c	1.1 ± 0.1	1.2 ± 0.6	1.1 ± 0.1	1.7 ± 0.5	1.1 ± 0.1	1.5 ± 0.3	1.7 ± 0.3	1.7 ± 0.4
YGM0d	3.9 ± 0.9	3.4 ± 0.3	3.0 ± 0.4	3.8 ± 0.7	4.3 ± 1.1	3.3 ± 1.3	3.3 ± 0.3	3.4 ± 1.0
YGM0e	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.9 ± 0.5	0.9 ± 0.2	0.8 ± 0.1
YGM0f	1.8 ± 0.1	1.6 ± 0.3	1.5 ± 0.1	1.7 ± 0.3	2.0 ± 0.4	1.6 ± 0.2	1.8 ± 0.5	2.7 ± 0.4
YGM0f'	29.4 ± 1.3	32.9 ± 1.9	30.8 ± 0.6	29.5 ± 2.1	30.6 ± 0.1	29.3 ± 1.2	32.8 ± 0.8	12.3 ± 1.4
YGM0g	8.2 ± 2.1	6.6 ± 0.5	8.4 ± 1.1	8.2 ± 1.8	11.3 ± 2.3	10.6 ± 0.6	10.5 ± 0.6	8.7 ± 1.0
YGM0g'	11.1 ± 1.5	12.3 ± 1.1	11.4 ± 1.2	10.3 ± 1.8	10.4 ± 0.4	10.9 ± 3.5	9.7 ± 2.4	3.0 ± 0.3
YGM0i	3.4 ± 0.3	2.8 ± 0.6	3.5 ± 0.3	3.2 ± 0.3	4.6 ± 1.0	4.9 ± 1.1	4.6 ± 0.6	2.9 ± 0.3
YGM1a&b	0.7 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.3	0.6 ± 0.2	1.3 ± 0.1	1.0 ± 0.2
YGM2	1.0 ± 0.5	0.5 ± 0.1	0.9 ± 0.4	0.8 ± 0.2	1.0 ± 0.2	1.1 ± 0.4	0.8 ± 0.2	4.6 ± 0.9
YGM3&3'	3.5 ± 0.8	3.4 ± 0.8	4.4 ± 0.4	3.7 ± 1.4	4.4 ± 0.6	5.8 ± 1.4	5.8 ± 0.7	3.0 ± 0.1
YGM5a&b	1.9 ± 0.1	2.2 ± 0.3	1.9 ± 0.5	2.1 ± 0.5	2.1 ± 0.5	2.6 ± 0.6	2.7 ± 0.6	1.8 ± 0.3
YGM6	1.7 ± 0.1	1.8 ± 0.5	2.1 ± 0.1	1.8 ± 0.7	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.3	0.6 ± 0.1
YGM7a	10.5 ± 2.8	14.6 ± 3.8	14.6 ± 1.9	11.5 ± 2.8	9.9 ± 1.2	11.0 ± 1.4	12.7 ± 2.2	0.8 ± 0.1
YGM7e	3.3 ± 1.4	4.8 ± 2.0	5.1 ± 0.8	3.3 ± 0.5	3.2 ± 0.5	2.9 ± 0.4	3.1 ± 0.6	T

* Treatment with the addition of K^+ (KCl) to the level of MS medium (20.05mM).

[a] Standard deviation of 3 independent determinations.

T:peak area less than 0.5%.

ND: not detected.

of pigment extract increased respectively from 11.0 ± 0.5 to 15.6 ± 2.0 (**Fig. 2A**). The decrease of NH_4NO_3 level from 20 to 10 mM slightly increased the colour value of pigment extract: from 9.1 ± 0.3 to 11.0 ± 0.5, respectively. Similarly, enhanced accumulation of anthocyanins was pronounced at the lowest levels of KNO_3 in comparison to their accumulation in MS medium (**Fig. 2B**). In this reaction to the decrease of nitrogen level in the medium, PL suspension culture resembled other cultures, such as grape (Hirasuna *et al.*, 1991), cranberry (Madhavi *et al.*, 1995) and *Vitis vinifera* L. (Do and Cormier, 1991a, 1991b).

The addition of K^+ ion in the treatments with decreased KNO_3 level in order to maintain its concentration at that of basal MS medium (20.05 mM) had no effect on anthocyanin accumulation (**Fig. 2B**). The same response was reported for grape suspension culture (Hirasuna *et al.*, 1991).

The decrease of NH_4NO_3 and KNO_3 concentrations in culture medium below their level in MS medium (which is 20.6 mM and 18.8 mM, respectively) up to 2.5 mM NH_4NO_3 and 9.4 mM KNO_3 didn't suppress the cell growth. However further decreases of their concentrations were unfavourable (**Fig. 2A** and **2B**). Pronounced culture growth at low nitrogen level in medium is concomitant with low nitrogen requirement for pigment production, which is a valuable characteristic of the PL suspension culture. Similarly, the growth of anthocyanin accumulating *Aralia cordata* cells cultured in the dark was reported to be promoted by 1/5 total nitrogen of the standard MS medium (Sakamoto *et al.*, 1993). In the contrary, the cell growth of *Vitis vinifera* suspension culture was greatly decreased in the medium with lower nitrates (Do and Cormier, 1991b).

A high growth index of 4.2(0.8 of PL suspension culture was recorded at presence of 56.4 mM KNO_3 (**Fig. 2B**). The design of our experiment doesn't suggest any reasonfor that. However, Chee *et al.* (1992) reported that embryogenic callus production of sweet potato "White Star" was doubled on basal callus proliferation medium modified to contain 60 mM K^+. PL callus culture possesses an embryogenic character (Konczak-Islam *et al.*, 1999). Therefore, it could be suspected that similar high level of K^+ might have contributed toward an enhanced growth of PL suspension culture.

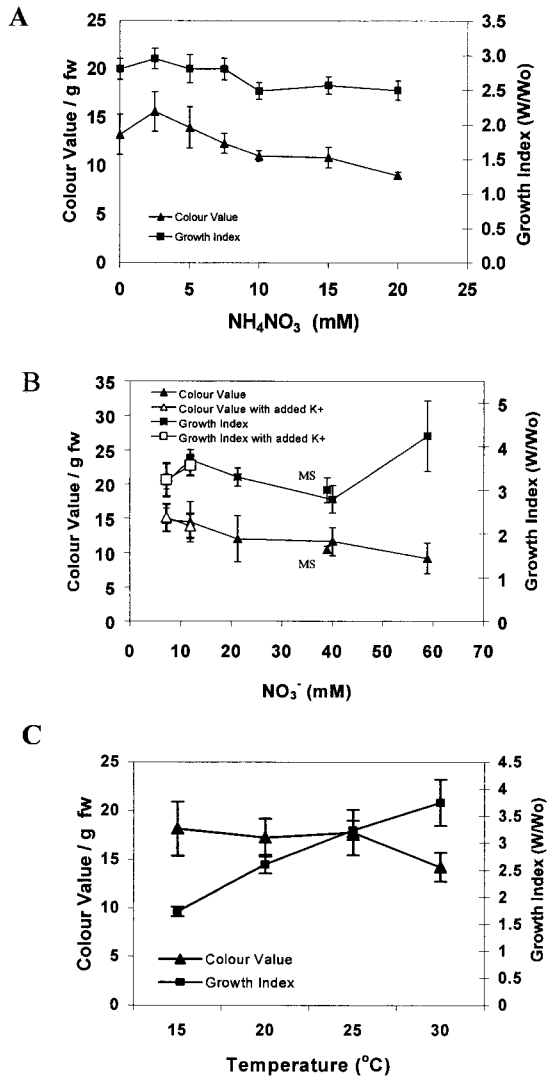

Fig. 2 The effect of NH_4NO_3 (A), NO_3^- (B) and temperature (℃) on cell growth and pigment accumulation in the PL suspension culture. Bars in the figure represent standard deviations of 4 replications.

Effects of temperature on pigment accumulation and cell growth.

The pigment composition of PL suspension culture incubated at 30 ℃ appeared to be significantly different to that of cell cultures at 15, 20 and 25 ℃ . At 30 ℃ the cells accumulated equal amount of YGM-0a, a non-acylated cyanidin, and YGM-0f', an acylated cyanidin (**Table 3**). The relative concentration of YGM-0a at 30 ℃ was at least twice as much as its concentration in cells exposed to lower temperature regimes. At the same time the relative concentrations of the pigments YGM-0f', -3' and -7a which appeared with later retention times were significantly less at 30 ℃ in comparison to their level at lower temperatures. No differences in pigment composition were detected among the PL suspension cultures incubated at 15, 20 and 25 ℃ . In these lower temperature treatments the dominant peak was an acylated YGM-0f'. Its relative concentration was about 3 times higher than that of the non-acylated YGM-0a. This result clearly indicates that the high temperature of 30 ℃ suppressed accumulation of metabolically advanced pigments, which appear on ODS-column HPLC with later retention times. Interestingly, the changes in pigment composition did not occurred gradually with temperature increase from 15 to 25 ℃ , but relatively suddenly, with its change from 25 to 30 ℃ . Therefore, the temperature of 30 ℃ significantly inhibited acylation and maybe methylation of YGM-0a.

The colour value of pigment extract of the cells incubated at 15, 20 and 25 ℃ was higher than that at 30 ℃ (**Fig. 2C**). This indicates that lower temperatures induced a higher anthocyanin accumulation within the cells. Total anthocyanin production in a culture is a function of cell growth and pigment accumulation. With the increase of temperature from 15 to 30 ℃ the cell growth index significantly increased (**Fig. 2C**). The maximum anthocyanin production per flask was achieved at 25 ℃ (data not presented). In this aspect PL suspension culture reacted to the different temperature regimes similarly to other anthocyanin producing cultures, such as *Perilla frutescens* (Zhong and Yoshida, 1993) and strawberry (Zhang *et al.*, 1997). Our result supports that reported by Kobayashi *et al.* (1998) of a strong influence of temperature regime on the total anthocyanin accumulation in the field grown storage root of sweet potato, cultivar Ayamurasaki. The authors observed a highly negative correlation between the average soil temperature (within a range of 22 to 29 ℃) and colour value of pigment extract and concluded that soil temperature is one of the important factors promoting change in anthocyanin content in the storage root of purple-coloured sweet potato.

The above results indicate that temperature, as well as ammonium level in culture medium, are important factors regulating anthocyanin biosynthesis in PL suspension culture. Both factors influence not only the level of accumulated pigment but also the composition and can therefore be used as tools to control the final quality of pigment. Highly acylated anthocyanins possess a high thermostability (Brouillard, 1981), which makes them an important source of anthocyanin-based food colorants. Therefore our finding might be used for the development of an efficient tissue culture system for commercial production of natural food colorants.

Among other factors sugar concentration in culture medium has been reported to have significant effect on anthocyanin accumulation. An increase of sucrose level up to 5 - 7% stimulated the production of anthocyanins in *Euphorbia milli* (Yamamoto

Table 3. Anthocyanin composition of PL suspension culture induced from storage root of *Ipomoea batatas.* cv. Ayamurasaki after 21 days of culture at different temperature. The values in the table are percentage of total anthocyanin calculated from the peak area at 530 nm.

Peak	Temperature (℃)			
	15	20	25	30
YGM0a	9.2 ± 1.2*	6.2 ± 0.8	8.9 ± 2.4	20.4 ± 4.8
YGM0a'	0.7 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	1.1 ± 0.1
YGM0b	1.8 ± 0.3	1.6 ± 0.2	3.3 ± 0.7	8.9 ± 2.0
YGM0c	1.9 ± 0.4	1.6 ± 0.2	1.4 ± 0.2	2.0 ± 0.4
YGM0d	7.0 ± 0.2	6.4 ± 0.6	5.5 ± 0.5	5.5 ± 0.3
YGM0e	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.8 ± 0.2
YGM0f	1.1 ± 0.1	1.5 ± 0.2	1.8 ± 0.1	1.9 ± 0.3
YGM0f'	27.0 ± 2.4	29.1 ± 0.7	29.3 ± 1.7	22.2 ± 2.8
YGM0g	8.2 ± 1.0	7.0 ± 0.4	6.7 ± 1.0	8.6 ± 0.7
YGM0g'	4.5 ± 0.5	6.2 ± 0.6	9.9 ± 1.3	7.2 ± 1.5
YGM0i	1.8 ± 0.2	2.0 ± 0.2	2.6 ± 0.2	3.8 ± 0.5
YGM1a&b	2.3 ± 0.3	1.7 ± 0.3	0.9 ± 0.2	0.5 ± 0.1
YGM2	1.4 ± 0.3	0.8 ± 0.3	0.6 ± 0.1	0.7 ± 0.1
YGM3	2.9 ± 0.6	1.7 ± 0.3	1.0 ± 0.3	0.8 ± 0.2
YGM3'	6.3 ± 0.6	6.2 ± 1.0	3.1 ± 0.6	1.3 ± 0.2
YGM5a&b	2.9 ± 0.3	2.8 ± 0.1	2.1 ± 0.3	1.3 ± 0.3
YGM6	3.1 ± 0.4	2.7 ± 0.1	2.4 ± 0.6	1.7 ± 0.4
YGM7a	11.9 ± 1.6	15.1 ± 0.9	12.0 ± 2.2	5.6 ± 1.8
YGM7e	0.5 ± 0.3	3.2 ± 0.5	4.3 ± 1.1	1.9 ± 0.7

* Standard deviation of independent determinations

et al., 1989) and up to 9% in *Vitis* cell suspension culture (Yamakawa *et al.*, 1983). However, sucrose concentration above 4% inhibited cell division in carrot suspension culture (Ozeki and Komamine, 1985) and above 5% reduced cell growth and anthocyanin accumulation in *Aralia cordata* callus culture (Sakamoto *et al.*, 1993). We have examined the influence of sucrose concentration at 1, 3, 5, 7 and 9% on anthocyanin accumulation in PL suspension culture. The level of sucrose exhibited strong effect on the amount of pigment accumulated in PL suspension culture: the colour value of pigment extract increased from 13.4 ± 1.2 at 1% sucrose to 23.0 ± 1.3 at 5% sucrose in the medium. The concentration above 5% didn't increase pigment accumulation and suppressed cell growth. The increase of sucrose concentration from 3 to 5% did not influence composition of anthocyanins (data not presented). The concentration of 5% was applied in the experiment with culture growth at different temperature regimes.

Some other factors reported to affect anthocyanin accumulation *in vitro*, such as light condition (Zhong *et al.*, 1991), auxin concentration (Sakamoto *et al.*, 1993, Ozeki and Komamine, 1981), phosphorus (Yamakawa *et al.*, 1983), sulphate and iron (Yamamoto *et al.*, 1989) may also influence pigment composition. Used at different levels, they may contribute towards or suppress accumulation of metabolically advanced pigments through activation or inhibition of different steps of anthocyanin biosynthetic pathway, such as acylation and/or methylation.

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References

Brouillard, R., 1981. Origin of the exceptional color stability of the *Zebrina* anthocyanin. Phytochemistry, **20**: 143-145.

Chee, R. P., Lescovar, D. I., Cantliffe, D. J., 1992. Optimizing embryogenic callus and embryo growth of a

synthetic seed system for sweetpotato by varying media nutrient concentrations. J. Amer. Soc. Hort. Sci., **117**: 663-667.

Do, C. B., Cormier, F., 1991a. Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape cell suspension culture. Plant Cell Rep., **9**: 500-504.

Do, C. B., Cormier, F., 1991b. Effects of high ammonium concentrations on growth and anthocyanin formation in grape (*Vitis vinifera* L.) cell suspension cultured in a production medium. Plant Cell Tiss. Org. Cult., **27**: 169-174.

Dougall, D. K., LaBrake, S., Whitten, G. H., 1983. Growth and anthocyanin accumulation rates of carrot suspension cultures grown with excess nutrients after semicontinuous culture with different limiting nutrients at several dilution rates, pHs, and temperatures. Biotechnol. Bioeng., **25**: 581-594.

Hirasuna, T. J, Shuler, M. L., Lackney, V. K., Spanswick, R. M., 1991. Enhanced anthocyanin production in grape cell cultures. Plant Sci., **78**: 107-120.

Hodgman, C. D., 1954. Handbook of Chemistry and Physics, 36th edition, Chemical Rubber Publishing Co., Cleveland, Ohio: 1617.

Kobayashi, T., Ikoma, H., Mochida, H., 1998. Effect of cultural conditions on anthocyanin content of purple-colored sweetpotato. Sweetpotato Res Front (KNAES, Japan), **6**: 2.

Konczak-Islam, I., Yoshinaga, M., Nakatani, M., Yamakawa, O., 1999. Anthocyanins in suspension cultures originating from different parts of sweet potato plant. Biotechnologia (Polish Academy of Sciences), **3**: 143-154.

Konczak-Islam, I., Yoshinaga, M., Nakatani, M., Yamakawa, O., Terahara, N., 2000. Establishment and characteristics of an anhtocyanin-producing cell line from sweet potato storage root. Plant Cell Rep., **19**: 472-477.

Madhavi, D. L., Smith, M. A. L., Berber-Jim_nez, M. D., 1995. Expression of anthocyanins in callus cultures of cranberry (*Vaccinium macrocarpon* Ait). J. Food Sci., **60**: 351-355.

Mori, T., Sakurai, M., 1994. Production of anthocyanin from strawberry cell suspension cultures; effects of sugar and nitrogen. J. Food Sci., **59**: 588-593.

Morris, P., 1986. Regulation of product synthesis in cell cultures of *Catharanthus roseus*. Effect of culture temperature. Plant Cell Rep., **5**: 427-429.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays in callus tissue cultures. Physiol. Plant., **15**: 473-497.

Odake K., Hatanaka, A., Kajiwara, T., Muroi, T., Nishiyama, K., Yamakawa, O., Terahara, N., Yamaguchi, M., 1994. Evaluation method and breeding of purple sweet potato "Yamagawa Murasaki" (*Ipomoea batatas* Poir.) for raw material of food colorants. Nippon Shokuhin Kogyo Gakkaishi, **41**: 287-293.

Odake, K., Terahara, N., Saito, N., Toki, K., Honda, T., 1992. Chemical structure of two anthocyanins from purple sweet potato, *Ipomoea batatas*. Phytochemistry, **31**: 2127-2130.

Odake, K., 1997. Characteristics of food color pigments derived from Ayamurasaki. In: LaBonte, D.R., Yamashita, M., Mochida, M. (Eds.): Proceedings of International Workshop on Sweetpotato Production System toward the 21st Century, 303-309. KNAES, Japan.

Ozeki, Y., Komamine, A., 1981. Induction of anthocyanin synthesis in relation to embryogenesis in a carrot suspension culture: Correlation of metabolic differentiation with morphological differentiation. Physiol. Plant., **53**: 570-577.

Ozeki, Y., Komamine, A., 1985. Effects of inoculum density, zeatin and sucrose on anthocyanin accumulation in a carrot suspension culture. Plant Cell Tiss. Org. Cult., **5**: 45-53.

Sakamoto, K., Iida, K., Sawamura, K., Hajiro, K., Asada, Y., Yoshikawa, T., Furuya, T., 1993. Effects of nutrients on anthocyanin production in cultured cells of *Aralia cordata*. Phytochemistry, **33**: 357-360.

Shimizu, T., Nakamura, M., 1993. Purple sweet potato color. In: Fuji, M., (Ed.): Natural Food Colorants, 224-225. Kohrin Corporation, Tokyo, Japan.

Shimomura, K., Sudo, H., Saga, H., Komada, H., 1991. Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. Plant Cell Rep., **10**: 282-285.

Terahara, N., Konczak-Islam, I., Nakatani, M., Yamakawa, O., Honda, T., 2000. *p*-Coumarylated anthocyanin in callus induced from the storage root of purple sweet potato. Phytochemistry, **54**: 919-922.

Terahara, N., Shimizu, T., Kato, Y., Nakamura, M., Maitani, T., Yamaguchi, M., Goda, Y., 1999. Six diacylated anthocyanins from the storage roots of purple sweet potato, *Ipomoea batatas*. Biosci. Biotechnol. Biochem., **63**: 1420-1424.

Yamakawa, T., Kato, S., Ishida, K., Kodama, T., Minoda, Y., 1983. Production of anthocyanins by *Vitis* cells in suspension culture. Agric. Biol. Chem., **47**: 2185-2191.

Yamamoto, Y., Kinoshida, Y., Watanabe, S., Yamada, Y., 1989. Anthocyanin production in suspension cultures of high-producing cells of *Euphorbia millii*. Agric. Biol. Chem., **53**: 417-423.

Yoshikawa, T., 1997. Production of ginsenosides in ginseng hairy root cultures. In: Doran, P.M.(Ed.): Hairy Roots: Culture and Applications, 73-79, Harwood Academic Publishers, Amsterdam.

Zhang, W., Seki, M., Furusaki, S., 1997. Effect of temperature and its shift on growth and anthocyanin production in suspension cultures of strawberry cells. Plant Sci., **127**: 207-214.

Zhong, J., Seki, T., Kinoshita, S., Yoshida, T., 1991. Effect of light irradiation on anthocyanin production by suspended culture of *Perilla frutescens*. Biotechn. & Bioeng., **38**: 653–658.

Zhong, J., Yoshida, T., 1993. Effects of temperature on cell growth and anthocyanin production in suspension cultures of *Perilla frutescens*. J. Ferment. Bioeng., **76**: 530–531.