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Induction of Betacyanin Synthesis and Pigment Accumulation in Cell Suspension Cultures of *Portulaca*

Naonobu NODA and Taiji ADACHI*

Applied Genetics and Biotechnology Division, Faculty of Agriculture, Miyazaki University, Miyazaki, 889-2192, Japan

** Present address: Department of Plant Science, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka, 599-8531, Japan.*

**Corresponding author* E-mail address : taijiada@plant.osakafu-u.ac.jp

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Abstract

The establishment of betalain-producing cell suspension cultures in *Portulaca* is reported for the first time. Addition of ascorbic acid (AsA) and sufficient osmotica to the culture medium was critical to the success. Betacyanin production was best at 1×10^3 mg l^{-1} AsA. Small cell aggregates, formed in MS-AsA medium supplemented with 150 mM mannitol and 150 mM sorbitol, accumulated the highest relative amounts of betacyanins. The growth rate of cell suspension cultures increased 3- to 4-fold within 14 days in modified MS-AsA medium supplemented with 5 μM 2,4-D. The betalain content of suspended cells increased during the first 2 day in liquid culture, thereafter decreased slightly and increased again during the logarithmic phase of cellular growth. A lower concentration of 2,4-D inhibited the growth of cultures but did not significantly affect betacyanin accumulation. Addition of cytokinin inhibited growth and also resulted in decreased betacyanin accumulation. The pigmentation of cultured cells decolourized upon transfer to dark conditions but was regained in decolourized cells at 12 to 24 hours after light exposure. The levels of endogenous free tyrosine and DOPA, precursors of betalains, decreased in response to light and slightly increased again after 24 hours of light treatment. Betanin, the main pigment component, as well as other betacyanins present, dramatically increased after 24 hours under light conditions in our *Portulaca* cell suspension culture system.

1. Introduction

In higher plants, the presence of betalain pigments is strictly confined to a small number of families, all of which are member of the order Caryophyllales, which lacks anthocyanins. Previous studies have shown that cell suspension cultures can be a useful tool for analyzing the regulatory mechanism of betalain biosynthesis and for the characterization of enzymes involved. Betacyanin synthesis was shown to be enhanced *in vitro* by 2,4-D (Sakuta *et al.*, 1991), and to be correlated with cell division activity (Hirano *et al.*, 1994) in cell suspension cultures of *Phytolacca americana*. Acyltransferase and glucosyltransferase involvement in betalain biosynthesis was studied in protein preparation from cell suspension cultures of *Chenopodium rubrum* (Bokern and Strack, 1998; Bokern *et al.*, 1991) and *Dorotheanthus bellidiformis* (Heuer and strack, 1992; Heuer *et al.*, 1996), respectively.

The genus *Portulaca* is especially well suited for the study of betalains. The genes encoding the enzymes required for the synthesis of betalains have been investigated by genetic analysis of flower colour (Ikeno, 1921; Adachi *et al.*, 1985; Trezzini and Zryd, 1990). Betalain accumulation has been demonstrated in cultured cells of *P. grandiflora* (Schröder and Böhm, 1984; Trezzini and Zryd, 1990) and *Portulaca sp.* 'Jewel' (Adachi, 1970; Kishima *et al.*, 1991) by using callus cultures. However, betalain producing cell suspension cultures have not been introduced to date. Betalain-synthesizing calli, when transferred to liquid medium, showed reduced growth with subsequent subcultures and eventually turned brown in colour and died (Böhm *et al.*, 1991). Suspension cultures of *P. oleracea* subsp. *sativa* proved to be easily initiated and maintained but the cells remained colourless (Böhm and Böhm 1996). In the present study, we used the variety 'Jewel' of *Portulaca* sp., a genotype that can be induced to produce betalain in callus cultures (Adachi, 1970; Kishima *et al.*, 1991), to establish cell suspension and, for the first time,

induced vigorous betalain synthesis in such fast-growing suspended *Portulaca* cells.

2. Materials and Methods

2.1 Plant material and culture conditions

Suspension cultures of *Portulaca* sp. 'Jewel' (inbred line JR) were initiated from callus. Callus cultures were initiated from seedlings as previously described (Kishima *et al.*, 1991). Callus were selectively subcultured at 25-day-intervals on MS medium (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and on J1 medium (Girod and Zryd, 1991) supplemented with 4.5 μM 2,4-D and solidified with 2.5 g l^{-1} gellan gum. Calli were transferred to liquid B5 (Gamborg *et al.*, 1968), MS and J1 medium supplemented with 5 μM 2,4-D 2,4-D and 20 g l^{-1} sucrose. Suspension cultures were maintained on a rotary shaker (100 rpm) at 25 ℃ in the light (photon flux density = 33.3 μmol m^{-2} s^{-1}) and subcultured at 10 days intervals. All media were adjusted with KOH and HCl to pH 5.7. The media containing AsA were filter sterilized (pore size: 0.22 μm).

2.2 Growth rate of the cell cultures

The fresh weight (wet weights) of the cultures was measured and averaged for at least three cultures. Cell growth rate was defined as W / W0, where W0 and W denote wet weight at the start and the end of defined culture period, respectively.

2.3 Extraction and analysis of betalains

Cells were collected and stored at -30 ℃. For analysis, frozen cells were thawed and resuspended in cold 80 % MeOH (1 m*l* per 100 mg wet weight). The extract was centrifuged at 1,710 × g for 5 min and the supernatant were concentrated at 30 ℃ *in vacuo*. The concentrate was dissolved in water (1m*l* g^{-1} wet weight) and immediately subjected to HPLC for betalain analysis. A 150 × 4.6 mm Shimadzu STR ODS-II column was used and maintained at 35 ℃ in a Shimadzu CTO-10A column oven. Solvent system A was 5 % acetic acid, solvent B 50 % acetonitrile. Betalains were resolved using a linear gradient from 0 to 40 % B at 2% B per min with a flow rate 1 m*l* min^{-1}. Betacyanins were detected at 540 nm. Betalamic acid and betaxanthins were detected at 430 or 480 nm. Betaxanthin identification was achieved through co-chromatography using semi-synthetic betaxanthins (Trezzini and Zryd, 1991, Toyama *et al.*, 1992).

2.4 Vis spectroscopy and quantification

Spectra and absorbances were determined through visible absorption with a Shimadzu multipurpose spectrophotometer (MPS-2000). The content of betacyanins and betaxanthins was calculated using the molar extinction coefficient (Girod and Zryd, 1991).

2.5 Extraction and analysis of free amino acid

Fifty mg of lyophilized cells was resuspended in 0.1 N HCl (10 m*l* g^{-1}). After centrifugation at 20,000 × g for 10 min, one volume of phenol / chloroform was added to the supernatant, vortexed and centrifuged at 20,000 × g for 10 min. One volume of diethylether was added to the aqueous phase and vortexed for 1 min. After centrifugation at 20,000 × g for 30 sec, the aqueous phase was lyophilized. Amino acids were derivatized with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acids and then analyzed by HPLC. Analysis of PTC amino acids was performed as follows. A Wakosil-PTC column was used and maintained at 40 ℃. Solvent A was 3.6 mM sodium acetate buffer (pH 6.0) in acetonitrile and solvent B 24 mM sodium acetate buffer (pH 6.0) in acetonitrile. Amino acids were resolved by running a linear gradient from A to B of 0 to 70 % B at 3.5 % B per min with flow rate of 1 m*l* min^{-1}. Amino acids were detected at 254 nm.

3. Results

3.1 Effect of ascorbic acid on the initiation of suspension cultures

To inhibit browing of tissue, degradation of betalains and necrosis of cells at initiation, the effect of ascorbic acid (AsA) was examined. Cell suspension cultures were initiated from callus lines that are high in betacyanin production (JR12 and JR18). Initial attempts to initiate betacyanin-producing suspension cultures in B5 liquid medium failed due to remaining large cell aggregates and moreover, a fading of the betacyanin content coupled with increasing brown pigmentation. A significant reduction of browning and betalain degradation was observed in medium supplemented with AsA. In 1 × 10^3 AsA containing B5 medium, the betacyanin content of culture was 1.2-fold higher than in AsA free medium (data not shown). The effect of different AsA concentrations (0, 1 × 10, 1 × 10^2, 1 × 10^3, 1 × 10^4 mg l^{-1}) on betacyanin content and growth of freshly initiated cell cultures was examined in MS and J1 liquid medium. Betacyanin content and growth rates were measured at 10 days after initiation. As shown in **Fig. 1**, the

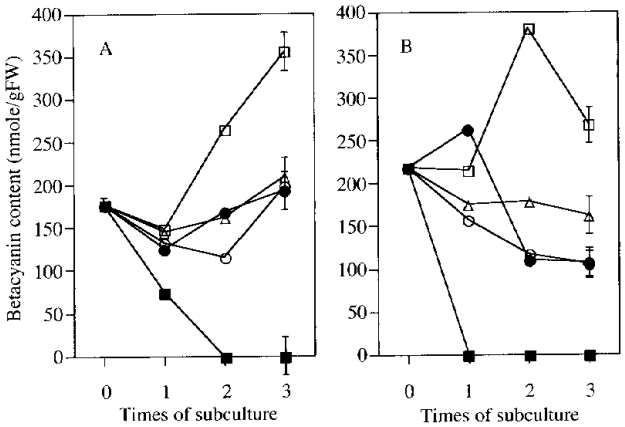

Fig. 1 Effects of ascorbic acid on accumulation of betacyanin in *Portulaca* cell suspension culture at initiation. Calli of JR12 line on MS (A) and J1 (B) solid medium were used as tested samples. Calli were transferred into liquid medium supplemented with several concentration (mg l^{-1}) of AsA (0= -○-, 1 × 10= -●-, 1 × 10^2= -△-, 1 × 10^3= -□-, 1 × 10^4= -■-). Vertical lines indicate SD (n=3).

betacyanins content reached a maximum at 1 × 10^3 mg l^{-1} AsA. Higher AsA concentrations resulted in reduced cell growth and completely suppressed betacyanin accumulation. Colourless cells also formed regularly in 0 to 1 × 10^2 mg l^{-1} AsA containing medium, while the culture growth was not significantly affected.

3.2 Effect of osmoticum on the initiation of suspension cultures

The effect of osmotic conditions on growth and betacyanin accumulation in freshly initiated suspension cultures was studied in MS-AsA (containing 1 × 10^3 mg l^{-1}) medium. Mannitol and sorbitol (1:1 w/w) as osmotica were used (Lu *et al.*, 1981). With increasing concentration of osmotica, slight decreases in growth of betacyanin producing cells were observed. Maximum betacyanin accumulation was found at 150 mM mannitol and 150 mM sorbitol in JR12-MS and JR12-J1 lines (**Fig. 2**), and at 50 mM mannitol and 50 mM sorbitol on JR18-MS line (data not shown). In JR12-MS line, no significant difference in betacyanin content was observed because of the grown large aggregates of calli were analysed. Only small cell aggregates, initiated from JR12-MS line, accumulated high amounts of betacyanins in MS-AsA supplemented with 150 mM mannitol and 150 mM sorbitol.

3.3 Characterization of growth and betacyanin accumulation in suspension cultures

Suspension cultures were fully established within

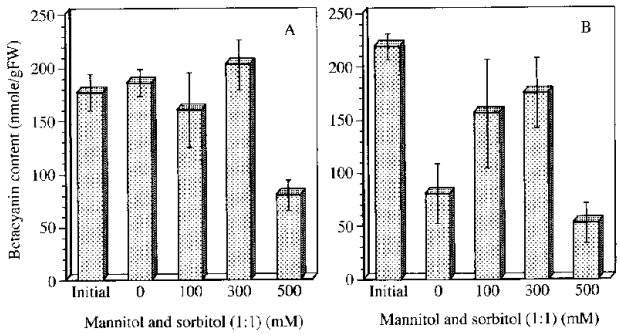

Fig. 2 Effects of osmoticum on accumulation of betacyanin in *Portulaca* cell suspension culture at initiation. Calli of JR12 line on MS (A) and J1 (B) solid medium were used as tested samples. Betacyanin contents were measured at day 10 after 2 cycles of subculture. Vertical lines indicate SD (n=3).

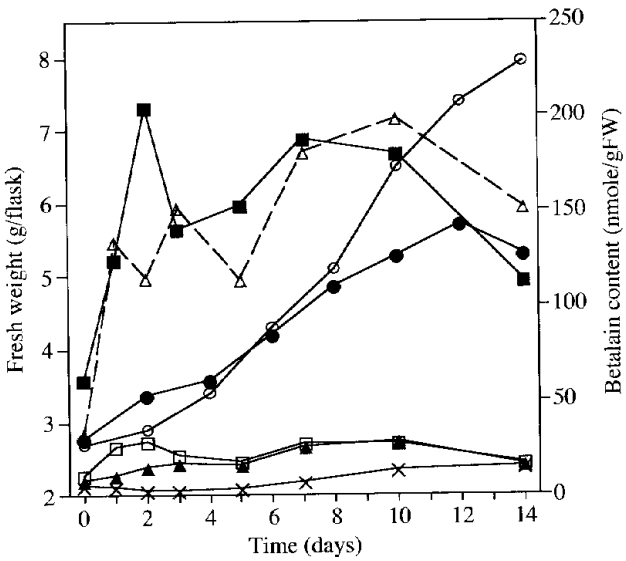

Fig. 3 Time courses of growth and betalain accumulation on modified MS-AsA medium in *Portulaca* cell suspension culture (JR12M7 line). Betanin (-■-), isobetanin (-□-), betanidin (-▲-) and acylated betacyanins (-△-) are betacyanin. Vulgaxanthin I (-×-) is betaxanthin. -○- = light condition. -●- = dark condition.

6 months after initiation. The cultures continuously maintained high productivity of the betacyanin for 3 years. Established cultures were subcultured in 10 day intervals by transfer of 3 packed cell volume into 25 m*l* of fresh medium in 100 ml Erlenmeyer flasks. The growth of cultures was more vigorous in MS-AsA medium than in J1-AsA medium. Under light condition, the cultures grew by 3- to 4-fold within 14 days after subculture (**Fig. 3**). Therefore, all cultures used for further analyses were grown and maintained on modified MS-AsA medium, which contained 1 × 10^3 mg l^{-1} AsA, 150 mM

mannitol and 150 mM sorbitol, supplemented with 5 μM 2,4-D. Betalain pigmentation immediately occurred after transfer to fresh medium. The betacyanin contents reached a first maximum on day 2, then decreased slightly and increased again on day 7 after subculture (**Fig. 3**). To confirm the effect of AsA and osmoticum, supplementary studies were carried out using 3-year-old suspension cultures (JR12-MS7 line). As shown in **Fig. 4**, addition of 1×10^3 mg l^{-1} AsA and 300 mM osmoticum were essential to accumulate betacyanins. In modified MS and J1 medium, the betacyanin content of cultures at 10 days after subculture was appoximately 1.6- and 1.8-fold higher than in AsA free medium, respectively (data not shown).

The influence of 2,4-D concentration on growth and betacyanin accumulation of suspension culture was also studied. 2,4-D concentrations suboptimal to cell growth did not significantly affect betacyanin accumulation in cultured cells (**Fig. 5**). At concentration higher than 50 μM, cells became necrotic. Cell aggregate size was enlarged and percentage of non-acylated betacyanin increased with decreasing 2,4-D concentration (data not shown). The effects of cytokinin on growth and betacyanin accumulation of suspension cultures on modified MS-AsA medium is presented in **Table 1**. Reduction of betacyanin accumulation was observed in both 5 μM 6-benzyladenine (6BA) and 5 μM kinetin (KIN) containing medium.

3.4 Betalain component in suspension cultures

In cell suspension cultures, approximately 2 times more betacyanins were accumulated than in callus cultures (**Fig. 1 and 4**). The cultures contained betanin (Rt 8.4 min) as the main component which accounted for up to 50% of all betacyanins. Minor components were isobetanin (Rt 10.0 min) at 10-15% and betanidin (Rt 11.2 min) at 5%. Four unknown compounds were detected after 20 min of retention time (**Fig. 6**). These betacyanins showed spectra typical of acylated derivatives, which absorb at approximately 550 nm and 330 nm (Strack and Wray, 1993). In the stem, pistil and stamen of *Portulaca sp.* 'Jewel', these acylated forms were found to be the main pigments. In the petals, however, the acylated betacyanins do not accumulate (**Fig. 6**). The cell suspension cultures produced predominantly betacyanin, while trace amount of betaxanthins, vulgaxanthin I and miraxanthin III were also detected (data not shown).

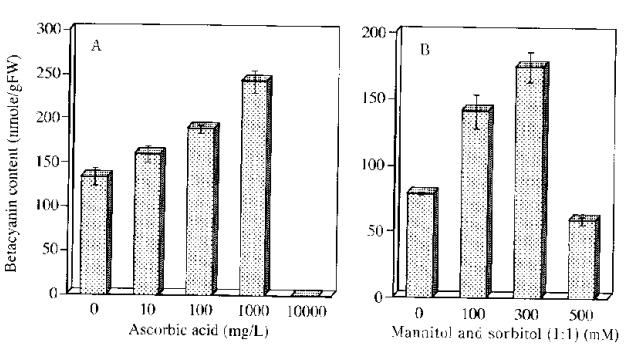

Fig. 4 Effects of ascorbic acid (A) and osmoticum (B) on accumulation of betacyanin in *Portulaca* cell suspension culture (JR12M7 line). Betacyanin contents were measured at 10 days after transfer. Vertical lines indicate SD (n=3).

3.5 The precursor and betalain accumulation after light irradiation

The amount of betalains and their precursors tyrosine and 3,4-dihydroxyphenylalanine (DOPA)

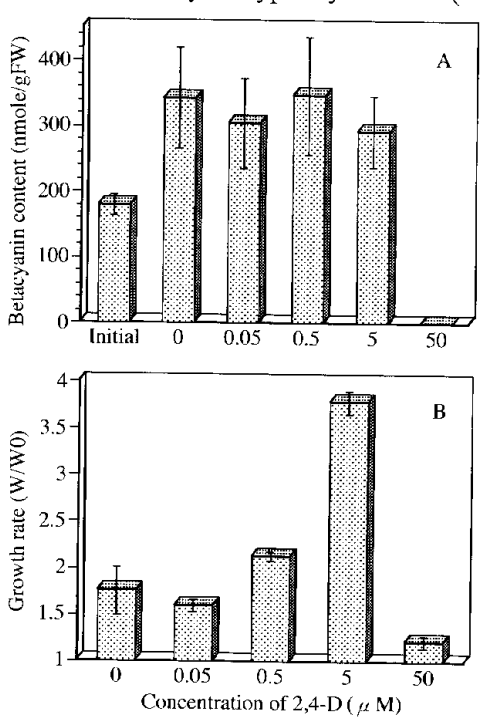

Fig. 5 Effects of various concentration of 2,4-D on betacyanin accumulation and growth rate of cultures. Betacyanin content and growth rate were measured at 10 days after transfer. A: Vertical lines indicate SD (n=5). B: Vertical lines indicate SD (n=3).

Table 1. Effect of cytokinins on the accumulation of betacyanin and growth rate of cell cultures.

Additions	Betacyanin content* nmole/gFW	Growth rate**
control	255.36 ± 20.48	4.65 ± 0.42
6BA 5×10^{-6}M	67.86 ± 19.05	2.41 ± 0.22
KIN 5×10^{-6}M	67.86 ± 11.23	2.90 ± 0.26

Notes: Fresh weight (FW) and betacyanin content were measured at 10 days after transfer. *SD (n = 5) **SD (n = 3).

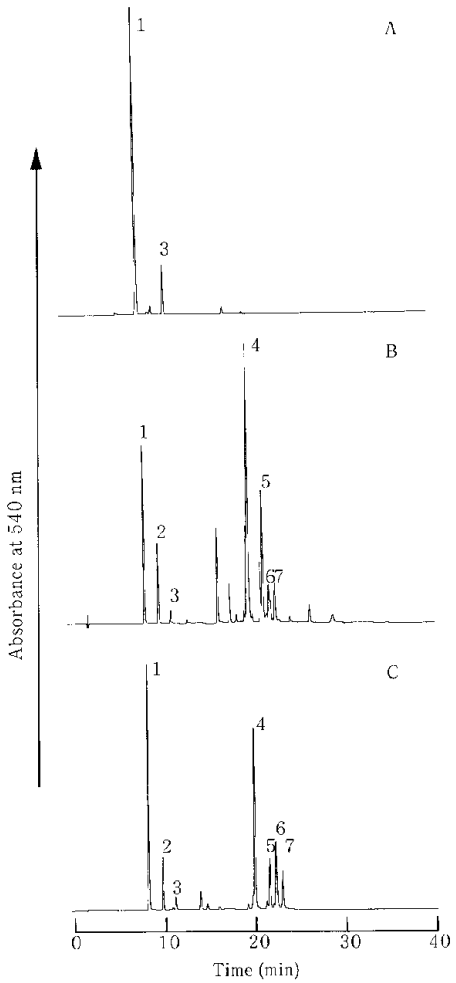

Fig. 6 Chromatograms of HPLC analyses of betacyanin from petals (A), stem (B), and cell suspension cultures (C) of *Portulaca* sp. 'Jewel' [inbred line JR (red flower)]. Betanin (1), isobetanin (2), betanidin (3), acylated betacyanin (4)-(7).

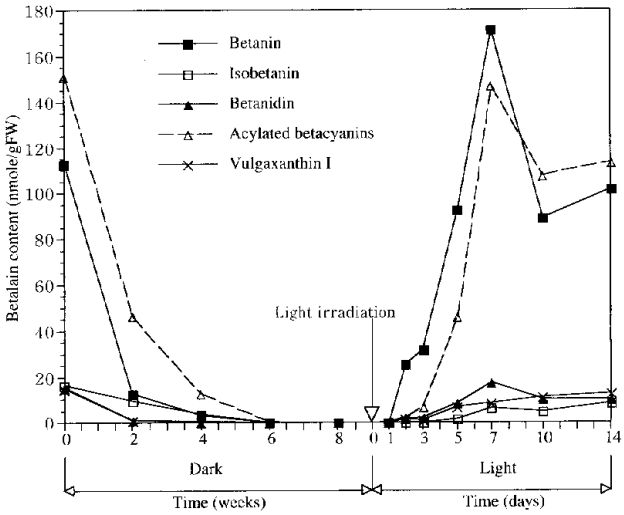

Fig. 7 Time courses of betalain accumulation in *Portulaca* cell suspension culture after dark and light treatment.

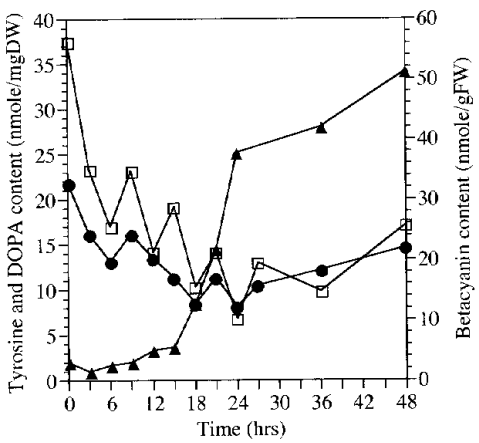

Fig. 8 Time courses of tyrosine and betacyanin accumulation after light irradiation. Cell suspension cultures were transferred to light condition at subculture. -□-=tyrosine. -●-=DOPA. -▲-=betacyanin.

was determined by HPLC combined with co-chromatography. Betalains disappeared gradually and pinkish-white cells were produced when cultures were transferred to dark conditions. After 4 subcultures (8 weeks), almost all cells appeared colourless (**Fig. 7**). Upon transfer to light, these cells were capable to turn magenta again. Seven days after fourth subculture, pigmentation of cultures was observed with the naked eye within 12 to 24 hrs after light exposure. Betanin, the main pigment component, dramatically accumulated during the first 24 hrs under light (**Fig. 8**). Simultaneously, decrease of tyrosine and DOPA was observed after light exposure followed by a slight increase after 24 hrs (**Fig. 8**). These results suggest that expression of genes involved in betalain biosynthesis was activated within 24 hrs after light exposure and continued up to the stationary phase of growth.

4. Discussion

In this experiment, we succeeded, for the first time, to induce betalain production in cell suspension cultures of *Portulaca*. Browning of suspended calli, degradation of betalains and necrosis of cells during initiation of the suspension cultures were overcome by the addition of AsA to the culture medium (**Fig. 1 and 4**). The survival of cells depends in part on the balance between formation of free radicals and its reduction. In general, AsA scavenges the free radical oxygen species and prevents such injuries as peroxidization of lipids, degradation of pigments and inactivation of enzymes (Shigeoka, 1996). Furthermore, ascorbate and related enzymes can be of particular importance

in regulating growth and development in plants (Córdoba and Gonzáez-Reyes, 1994). AsA probably protected the cultures from oxidative stress during subculture to fresh and aerated liquid medium, which resulted in efficient growth and accumulation of betacyanins. The content of betacyanin decreased dramatically during the first days of culture in AsA-free medium. This is similar to the observations made in cell suspension cultures of *Chenopodium rubrum* (Berlin *et al.*, 1986) and *Phytolacca americana* (Sakuta *et al.*, 1986). In modified MS-AsA medium, however, betalain pigmentation occurred earlier than the logarithmic phase of growth in cell suspension cultures of *Portulaca* (**Fig. 3**). Therefore, it is possible that AsA is also involeved in a specific function in betalain synthesis. Reynoso *et al.* (1997) reported that ascorbic acid counteracted oxidation of betalain and protected its colour stability *in vitro*. AsA led to the reduction of dopachrome to cyclo-dopa (Schliemann *et al.*, 1998) and inhibited the oxidation activity of the tyrosinase *in vitro* (Steiner *et al.*, 1999). More experiments are necessary to determine the role of AsA in our cultures and to clarify the possible function of AsA in betalain biosynthesis *in vivo*.

Betacyanin accumulation in *Portulaca* cell suspension cultures was stabilized and enhanced by addition of mannitol and sorbitol to the culture medium (**Fig. 2 and 4**). At the same time, small aggregates of compact cells were produced in MS-AsA medium supplemented with 58.4 mM (20 g l^{-1}) sucrose, 150 mM mannitol and 150 mM sorbitol. A similar correlation between cell size and betacyanin accumulation was reported previously for cell suspension cultures of *P. americana* (Sakuta *et al.*, 1987). Cell size decreased and relative betacyanin accumulation increased with decreasing water potential, caused by mannitol. Addition of mannitol to the culture medium also significantly increased the accumulation of anthocyanin in grapes (Chi and Cormier, 1990). In our experiments, a combination of mannitol and sorbitol, besides creating the optimal osmotic conditions for the cells, effected the accumulation of betalains.

Growth rate of suspended cells reached a maximum at 5 μM 2,4-D in *Portulaca*. Cell suspension cultures of *C. rubrum* (Berlin *et al.*, 1986) and *Beta vulgaris* (Leathers *et al.*, 1992) are capable of producing betacyanin in well growing cultures at low concentrations of 2,4-D. As with most cell suspension cultures, the betacyanin accumulation can be obtained through vigorous growth. Betacyanin content per fresh weight did not differ significantly with respect to 2,4-D concentrations (**Fig. 5**). This is in contrast with reported betacyanin accumulation in cell suspension cultures of *P. americana*, where, at concentration of 2,4-D lower than 1 μM, marked reduction in betacyanin accumulation with decreasing cell numbers was observed (Sakuta *et al.*, 1991). The cell suspension cultures of *Portulaca* may have the ablility to keep level of betacyanin accumulation under various stress conditions such as the suppression of cell division.

Cytokinins suppressed growth of cultures as well as betacyanin accumulation in *Portulaca* (**Table 1**). Studies in *Amaranthus tricolor*, however, have shown that kinetin induced betacyanin formation in seedlings (Piatteli *et al.*, 1971) and calli (Bianco-Colomas and Hugues, 1990). Treatment with 6-benzyladenine also increased betacyanin content in seedlings of *A. caucadus* (Féray *et al.*, 1992). In our system, cytokinins inhibited the growth of cultures, which resulted in a reduction of the accumulation of betacyanins. A Similar pigment response was reported for suspension cultures of *Phytolacca*, except that cytokinins did not affect the growth (Hirano *et al.*, 1992). Compared to *Phytolacca* cells, exogenously supplied cytokinins generate super-optimal conditions in *Portulaca* cells and further affect the primary metabolism.

The data reported here indicate that betacyanin accumulation in suspension cultures of *Portulaca* sp. 'Jewel' is stimulated by light with growth of cultures. Betalain pigmentation in *Portulaca* callus is efficiently induced by blue light combined with ultra-violet irradiation (Kishima *et al.*, 1995). In suspended cells, betacyanin formation continued even in dark grown cultures for 1 month (**Fig. 7**). When decolourized cultures were transferred to light conditions during the stationary growth phase, betacyanins synthesis was not induced. As with calli of *C. rubrum*, betacyanin could be induced in the dark (Berlin *et al.*, 1986). The expression of betacyanin synthesis genes is remarkably stimulated by light, however, cell division and growth of cultures are critical. The light-induced changes in gene expression and enzyme activity of the flavonoid metabolism were extensively studied in cell suspension cultures of parsley (Schröder *et al.*, 1979; Kreuzaler *et al.*, 1983) and carrot (Takeda *et al.*, 1993). Betalain accumulation is rapidly, within 1 day, induced by light irradiation in our *Portulaca* cell suspension cultures (**Fig. 7 and 8**). Subsequent differences in its regulation by light irradiation can provide some clue to the gene expression related betalain biosynthesis. The suspension culture system of *Portulaca*, inducible for betacyanin accumulation and introduced in this report, should be highly suitable for biochemical and molecular bio-

logical studies of gene expression and enzymes involved in betalain biosynthesis.

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References

Adachi, T., 1970. Studies of biochemical genetics on flower colour and its application to flower breeding. VI. Biogenesis of betalain pigment and variations of intracellular conditions in *Portulaca* callus. Bull. Fac. Agric. Miyazaki Univ., **17:** 143-150 (in Japanese).

Berlin, J., Sieg, S., Strack, D., Bokern, M., Harms, H., 1986. Production of betalains by suspension cultures of *Chenopodium rubrum* L.. Plant Cell Tissue Organ Cult., **5:** 163-174.

Bianco-Colomas, J., Hugues, M., 1990. Establishment and characterization of a betacyanin producing cell line of *Amaranthus tricolor*: Inductive effects of light and cytokinin. J. Plant Physiol., **136:** 734-739.

Bokern, M., Strack, D., 1988. Synthesis of hydroxycinnamic acid esters of betacyanins via 1-*O*-acylglucosides of hydroxycinnamic acids by protein preparations from cell suspension cultures of *Chenopodium rubrum* and petals of *Lampranthus sociorum*. Planta, **174:** 101-105.

Bokern, M., Heuer, S., Strack, D., 1992. Hydroxycinnamic acid transferases in the biosynthesis of acylated betacyanins: Purification and characterization from cell cultures of *Chenopodium rubrum* and occurrence in some other members of the Caryophyllales. Bot. Acta, **105:** 146-151.

Böhm, H., Böhm, L., 1996. *Portulaca grandiflora* Hook. and *P. oleracea* L.: Formation of betalains and unsaturated fatty acids. In: Y.P.S. Bajaj (Eds.): Medical and Aromatic Plants IX, Biotechnology in Agriculture and Forestry Vol.37., 335-354. Springer-Verlag, Berlin Heidelberg.

Böhm, H., Böhm, L., Rink, E., 1991. Establishment and characterization of a betaxanthin-producing cell culture from *Portulaca grandiflora*. Plant Cell Tissue Organ Cult., **26:** 75-82.

Chi, B. D., Cormier, F., 1990. Accumulation of anthocyanins enhanced by a high osmotic potential in grape (*Vitis vinifera* L.) cell suspensions. Plant Cell Rep., **9:** 143-146.

Córdoba, F., Gonzáez-Reyes, J. A., 1994. Ascorbate and plant cell growth. J. Bioenerg. Biomenbr., **26:** 399-405.

Féray, A., Hourmant, A., Penot, M., Moisan-Cann, C., Caroff, J., 1992. Effects of interaction between polyamines and benzyladenine on betacyanin synthesis in *Amaranthus* seedlings. J. Plant Physiol., **139:** 680-684.

Gamborg, O. L., Miller, R. A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cell. Exp. Cell. Res., **50:** 151-158.

Girod, P.-A., Zrÿd, J.-P., 1991. Secondary metabolism in cultured red beet (*Beta vulgaris* L.) cells: Differential regulation of betaxanthin and betacyanin biosynthesis. Plant Cell Tiss. Org. Cult., **25:** 1-12.

Heuer, S., Strack, D., 1992. Synthesis of betanin from betanidin and UDP-glucose by protein preparation from cell suspension cultures of *Dorotheanthus bellidiformis* (Burn. F.) N. E. Br.. Planta, **186:** 626-628.

Heuer, S., Vogt, T., Böhm, H., Strack, D., 1996. Partial purification and characterisation of UDP-glucose: betanidin 5-*O*- and 6-*O*-glucosyltransferases from cell suspension cultures of *Dorotheanthus bellidiformis* (Burm. F.) N. E. Br.. Planta, **199:** 244-250.

Hirano, H., Sakuta, M., Komamine, A., 1992. Inhibition by cytokinin of the accumulation of betacyanin in suspension culture of *Phytolacca americana*. Z. Naturforsh., **47c:** 705-710.

Hirano, H., Komamine, A., 1994. Correlation of betacyanin synthesis with cell division in cell suspension cultures of *Phytolacca americana*. Physiol. Plant., **90:** 239-245.

Ikeno, S., 1921. Studies on the genetics of flower-colours in *Portulaca grandiflora*. J. Coll. Agr. Imp. Univ. Tokyo, **8:** 93-133.

Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D. N., Hahlbrock, K., 1983. UV-induction of chalcone synthase mRNA in cell suspension culture of *Petroselinum hortense*. Proc. Natl. Acad. Sci. USA, **80:** 2591-2593.

Kishima, Y., Nozaki, K., Akashi, R., Adachi, T., 1991. Light-inducible pigmentation in *Portulaca* callus; selection of a high betalain producing cell line. Plant Cell Rep., **10:** 304-307.

Kishima, Y., Shimaya, A., Adachi, T., 1995. Evidence that blue light induces betalain pigmentation in *Portulaca* callus. Plant Cell Tiss. Organ. Cult., **43:** 67-70.

Lethers, R. R., Davin, C., Zrÿd, J.-P., 1992. Betalain producing cell cultures of *Beta vulgaris* L. var. bikores monogerm (red beet). In Vitro Cell. Dev. Biol., **28P:** 39-45.

Lu, C., Vasil, V., Vasil, I. K., 1981. Isolation and culture of protoplasts of *Panicum maximum* Jacq. (Guineagrass): somatic embryogenesis and plantlet formation. Z. Pflanzenphysiol., **104:** 311-318.

Murashige, M., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tabacco tissue cultures. Physiol. Plant., **15:** 473-497.

Piattelli, M., Giudici de Nicola, M., Castrogiovanni, V., 1971. The effect of kinetin on Amaranthin synthesis in *Amaranthus tricolor* in darkness. Phytochem., **10:** 289-293.

Reynoso, R., Garcia, F. A., Morales, D., Gonzalez de Mejia, E., 1997. Stability of betalain pigments from Cactacea fruit. J. Agric. Food. Chem., **45:** 2884-2889.

Sakuta, M., Takagi, T., Komamine, A., 1987. Effect of sucrose on betacyanin accumulation and growth in suspension cultures of *Phytolacca americana*. Physiol. Plant., **71:** 455-458.

Sakuta, M., Hirano, H., Komamine, A., 1991. Stimulation by 2,4-dichlorophenoxyacetic acid of betacyanin accumulation in suspension cultures of *Phytolacca americana*. Physiol. Plant., **83:** 154-158.

Schliemann, W., Steiner, U. and Strack, D., 1998. Betanidin formation from dihydroxyphenylalanine in a model assay system. Phytochem., **49:** 1593-1598.

Schröder, J., Kreuzaler, F., Schöder, E., Haulbrock, K., 1979. Concomitant induction of phenylalanine ammonia-lyase and flavone synthase mRNAs in irradiated plant cells. J. Biol. Chem., **254:** 57-65.

Schröder, W., Böhm, H., 1984. Betacyanin concentration in young cell cultures from *Portulaca grandiflora* - an analysis of variation. Plant Cell Rep., **3:** 14-17.

Shigeoka, S., 1996. Why is ascorbate present in large amounts in plant cells? Foods & Food Ingredients J. Jpn., **170:** 66-74 (in Japanese).

Strack, D. Wray, V., 1993. Recent Advances in Betalain Analysis. In: Behnke, H. D. and Mabry, T. J. (Eds): Caryophyllales: Evolution and systematics, 263-277. Springer Verlag.

Steiner, U., Schliemann, W., Böhm, H., Strack, D., 1999. Tyrosinase involved in betalain biosynthesis of higher plants. Planta, **208:** 114-124.

Takeda, J., Abe, S., Hirose Y., Ozeki, Y., 1993. Effect of light and 2,4-dichlorophenoxyacetic acid on the level of mRNA for phenylalanine ammonia-lyase and chalcone synthase in carrot cells culture in suspension. Physiol. Plant., **89:** 4-10.

Trezzini, G. F. Zrÿd, J.-P., 1990. *Portulaca grandiflora*: A model system for the study of the biochemistry and genetics of betalain synthesis. Acta Horticulturae, **280:** 581-585.

Trezzini, G. F., Zrÿd, J.-P., 1991. Characterization of some natural semi-synthetic betaxanthins. Phytochem., **30:** 1901-1903.

Toyama, J., Terahara, N., Adachi, T., 1992. Identification of betalain pigments and the changes of HPLC pattern during developmental stage in some *Portulaca* genotypes. Breeding Science, **48** (suppl. 2) : 330-331 (in Japanese).