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Sucrose and light induced betalain biosynthesis in callus cultures of bougainvillea (*Bougainvillea* spp.)

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ABSTRACT

An attempt was made to substantiate the optimum sucrose level and light conditions for biosynthesis of betalain pigments (betaxanthins and betacyaninins) from callus cultures of bougainvillea (*Bougainvillea* spp.). A sucrose concentration of 50 g/l in MS medium resulted in maximum response coefficient with earliest pigment initiation and intensification. Sucrose at 50 g/l resulted in significant increase in production of betacyanin and betaxanthin as compared to control. Under high sucrose concentration callus growth was significantly decreased. Continuous blue light was most effective in enhancing the betacyanin and betaxanthin content in the callus cultures. Under complete darkness there was significant decrease in pigment content but the callus growth was increased.

Key words: Bougainvillea, Callus cultures, Light, Pigment induction, Sucrose.

Betalains are water-soluble, nitrogen-containing plant pigments whose colours range from red-violet betacyanins to yellow betaxanthins (Tanaka *et al.* 2008). These pigments are found in the cell sap of plants representing most families of the Caryophyllales and some higher fungi belonging to the genera *Amanita* and *Hygrocybe.* Interest in these molecules has grown since their antioxidant and free radical scavenging properties were characterized (Kanner *et al.* 2001). Recently, it has been reported that betanin induces apoptosis in human chronic mylloid leukemia cells (Sreekanth *et al.* 2007). Hence, betalains are likely to be highly suitable in natural colourants for preparing healthy foods and their consumption is likely to increase. Plant cell and tissue cultures are attractive alternative sources of bioactive plant substances, including betalain pigments (Rao and Ravishankar 2002). *In vitro* system offers several advantages over field cultivation: it is independent of geographical and seasonal variations, environmental factors, and political interference; in addition, it allows optimal and stable growth conditions, voluntary modulation of growth parameters, and constant quality control (Rao and Ravishankar 2002; Moreno *et al.* 2008). It also eliminates negative biological influences (microorganisms and insects) that affect secondary metabolites production in nature; and possibility to select cultivars with higher production of secondary metabolites (Mulabagal *et al.* 2004).

Myrtillocactus geometrizans, *Portulaca grandiflora*, *Beta vulgaris*, *Chenopodium rubrum* and many other plants have been introduced in *in vitro* culture with the purpose of studying the biosynthesis and eventual commercial production of betalains. However, research on betalain biosynthesis under *in vitro* conditions in the ornamental plant bougainvillea (*Bougainvillea* spp.), which contains betalains, is still limited. Hence, the present study was undertaken with an aim to investigate the effect of various sucrose levels and light conditions on stimulation of betalain biosynthesis in bougainvillea callus cultures.

MATERIALS AND METHODS

Explant and callus induction

Callus used in this investigation was derived from leaf explant of bougainvillea cv. Bhabha cultured on the MS basal medium (Murashige and Skoog 1962) supplemented with 6 mg/l 2,4-D and it was continuously maintained on the same medium with double the quantity of vitamins at 24 ± 1°C in complete darkness. Stock callus cultures were maintained under the same physical conditions described above that were sub-cultured at 21-day interval.

Sucrose treatment for betalain induction

The different sucrose levels, *viz*. 30 (control), 40, 50 and 70g/l were incorporated in the MS medium and callus

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were cultured on these treatments. The pH of the medium was adjusted to 5.8 ± 0.1 prior to adding agar-agar (5.5 g l^{-1}, Qualigens), autoclaved (121°C) for 15 min and dispensed into test tubes (25 ml).Cultures were incubated in a culture room at 24 ± 1°C under 16/8 h (105.7 µmol photons $m^{-2}s^{-1}$ light/dark) photoperiod regime using cool-white fluorescent tubes. In another experiment, the callus cultures were subjected to four light conditions, *viz*. 16/8 h (control), complete darkness, continuous white light and continuous blue light to check their effectiveness in inducing pigmentation in the callus.

Callus growth and response coefficient

The betalain biosynthesis in the callus was measured by different parameters such as response coefficient = (total number of cultures showing pigmentation/ total number of cultured cultures)× 100, number of days taken for pigment initiation and intensification which was visually observed and callus biomass accumulation which was measured by determining the fresh cell weight (FCW) of callus after 28 days of culture.

Betalain estimation

Extraction and quantification of betalains from bougainvillea callus tissue was carried out based on the method described by Castellanos-Santiago and Yahia (2008) with minor modifications. Proliferated callus masses were harvested to measure the betacyanin and betaxanthin content. Pigmented and non-pigmented callus samples (100 mg) were macerated using double-distilled water. The extracts were centrifuged at 12000 × g for 10 min. in a refrigerated (4°C) centrifuge (Sigma 3K30, Germany). Optical density (OD) of the supernatant of each sample was measured at 483 and 535 nm using a UV-Vis double-beam spectrophotometer (Thermo Electron Corp, USA) against the blank which consisted of double-distilled water.

Statistical analysis

The experiment was laid out in completely randomized design (CRD). The data were subjected to analysis of variance and significance was assumed at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of sucrose levels on biosynthesis of betalain

The manipulation of the culture environment can be effective in enhancing secondary metabolite production, once the biosynthetic pathways are easily altered by external factors such as nutrient levels, plant growth regulators and stress factors (Rao and Ravishankar 2002). The results presented in Table 1 revealed that when the callus were subjected to different sucrose concentrations [30 (control), 40, 50 and 70 g/l] in MS medium the response coefficient varied among the calluses. Callus cultured on 50g/l sucrose supplemented medium significantly differed ($P \leq 0.05$) and gave maximum response coefficient (73.75%).With this sucrose level the number of days taken for pigment initiation and intensification was also significantly lower. However, callus cultured on the control took maximum days to pigment initiation and initiation. At higher sucrose concentration there was a significant decrease in response coefficient.

Table 1 Effect of sucrose levels on betalain biosynthesis in bougainvillea callus cultures

Treatment (g/L)	Response coefficient (%)	No. of days taken for pigment	
		Initiation	Intensification
30 (control)	61.25	14.75	23.25
40	66.25	10.75	18.25
50	73.75	9.25	16.75
70	57.50	13.00	22.50
CD (P=0.05)	6.05	1.29	1.51

With the increase in sucrose concentration biosynthesis of betacyanins and betaxanthins also increased and it was maximum in medium supplemented with 50 g/l sucrose (Fig 1). In MS medium with 50 g/l sucrose the betacyanin content was 0.44 mg/g FW while betaxanthin content was 0.31 mg/g FW. At higher concentration the pigment production however decreased in the cultures.

This reduction may be due the fact that elevated sucrose levels caused high osmotic stress in the culture medium and in response to which the uptake of nutrients might have decreased. Further, these results showed that biosynthesis of betalain pigments in bougainvillea callus was associated with the supplementation of sucrose concentration in the MS medium. Many plant genes are controlled by sugars that are involved in a variety of processes such as photosynthesis, storage of protein/starch/lipid and production of homo- and hetero-polysaccharides (Gibson 2000). Sugars are also known to interact with several growth regulators leading to the changes in the array of morphological events (Kraemer *et al.* 2002).

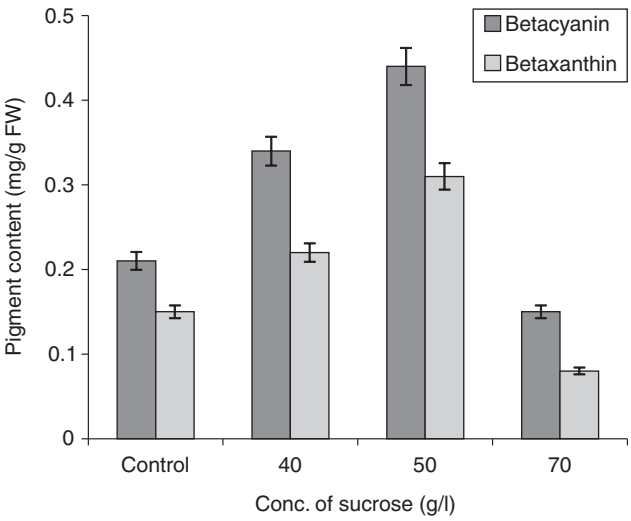

Fig 1 Effect of sucrose levels on betalain biosynthesis.

Results suggested that the addition of sucrose up to an optimum level in the culture medium showed an up-regulated betalain biosynthesis in the callus cultures. The results are consistent with the findings of Akita *et al.* (2002). For this, several hypotheses are possible. The first hypothesis is that sucrose, a source of energy, is required as substrates for betalain biosynthesis. The second hypothesis is that sucrose increases the osmotic potential of culture medium which imposes the osmotic stress in the cultures, thereby inducing the betalain biosynthesis.

Whether the sucrose controlled betalain biosynthesis in bougainvillea is affecting the callus biomass accumulation or not, the gain in the fresh cell weight was investigated. It is evident from the Fig 2 that as the level of sucrose in the culture medium increased from 30 to 70g/l the growth of callus tissues was significantly decreased. Therefore, relationship between callus growth and increase in sucrose level in culture medium was found to be inversely related to each other. The maximum growth of calluses in term of biomass accumulation was recorded in control, i.e. MS medium only. Thus, high sucrose level adversely affected the callus biomass accumulation which may be linked to the high osmotic stress brought about by high sucrose level in the culture medium which may have resulted in lesser uptake of nutrients and thereby, reduced callus biomass accumulation. This result is in agreement with findings of Sato *et al.* (1996) in which they pointed out that the decrease of cell growth in media containing a high sucrose concentration might have been caused by inhibition of nutrient uptake in strawberry suspension culture due to an increase in the osmotic potential or high viscosity of the medium.

Besides this, it was also reported that depletion of some nutrients lead to enhancement of secondary metabolites, but with growth limitations (Narayan *et al.* 2005). Outcome of this experiment confirmed that higher level of sucrose supplementation into the culture medium increases the osmotic potential which subsequently leads to reduction in the nutrient uptake and thereby poor callus biomass accumulation. Therefore, the optimum sucrose level proved to be an important factor for modulating betalain biosynthesis in callus cultures of bougainvillea cv. Bhabha.

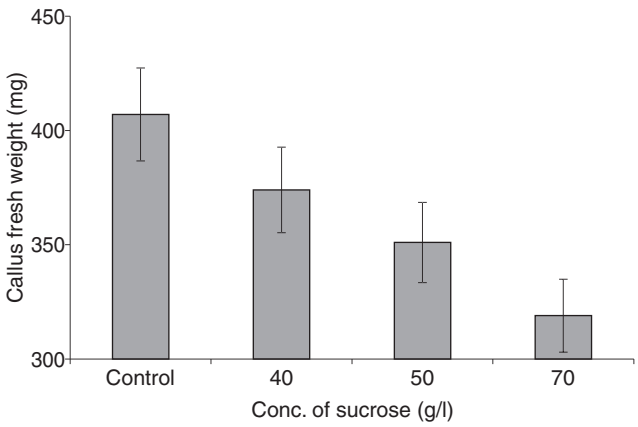

Fig 2 Effect of sucrose levels on callus fresh weight.

Table 2 Effect of light conditions on betalain biosynthesis in bougainvillea callus cultures

Treatment	Response coefficient	No. of days taken for pigment	
	(%)	Initiation	Intensification
16/8 h (control)	61.25	14.75	23.25
Complete darkness (CD)	47.50	18.25	27.50
Continuous white light (CWL)	71.25	11.50	19.25
Continuous blue light (CBL)	80.00	8.50	15.50
CD (P=0.05)	3.56	1.06	1.23

Effect of light conditions on biosynthesis of betalains

In the present study, light treatments were found to have an effect on response coefficient, number of days required for pigment initiation and intensification (Table 2). The maximum response coefficient in callus (80%) was observed under continuous blue light conditions which was significantly higher than all the treatments ($P \leq 0.05$). Under complete darkness there was a significant reduction in response coefficient. Earliest pigment initiation (8.50 days) and intensification (15.50 days) in the cultures was recorded under continuous blue light conditions. The response coefficient under continuous white light was also better though it was less as compared to continuous blue light conditions. When cultures were grown on MS medium under complete darkness they took maximum number of days for pigment initiation and intensification. Under continuous blue light conditions, there was significant increase in betalain (betacyanin and betataxanthin) content in the calluses (Fig 3). The betacyanin content was recorded to be 0.57 mg/g FW and the bexanthin content was 0.45 & 0.30 mg/g FW when the callus cultures were subjected to continuous blue light conditions. This was significantly higher than control. Under continuous white light also there was increase in pigment content but it was lower as compared to continuous blue light conditions. When the callus cultures were subjected to complete darkness there was a significant reduction in betalain content (betacyanin and betaxanthin).

To ascertain the effect of different light conditions on callus biomass accumulation, the gain in fresh cell weight was investigated. It was observed that there was reduction in growth of callus tissues under continuous white light and blue light conditions as compared to control. However, under complete darkness there was increase in growth of callus tissues.

Plant responses to red, blue and UV radiation evoke different expression *via* signal perception and transduction pathways (Kendrick and Kronenberg 1993). Most of *in vitro*

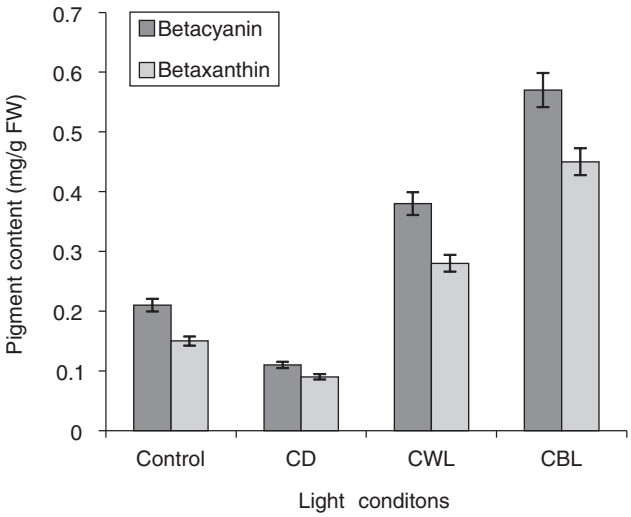

Fig 3 Effect of light conditions on betalain biosynthesis.

studies show some effect of radiation on the accumulation of betalain in cultured cells (Bianco-Colomos and Huges 1990; Bohm *et al.* 1991) although details of responses have not yet been clarified. Blue radiation was more effective than red radiation in inducing betalains in seedlings of *Amaranthus* (Obrenovic 1985) and callus cultures of *Portulaca* (Kishima *et al.* 1995). In the present study also blue light has induced greater response coefficient and betalain synthesis than other light treatments. Photomorphogenesis in higher plants is under the control of at least three different photo-transduction system involving phytochrome, blue radiation, and UV photoreceptors (Kendrick and Kronenberg 1993). In this study, betalain biosynthesis in callus cultures irradiated with continuous blue light was most efficient among the four light sources employed. Thus, betalain pigmentation in the callus could be activated through blue light signal transduction where flavin-like photoreceptors are involved in the initiation affecting certain gene expression (Ahmad and Cashmore 1993). Bhuyian *et al.* (2002) reported that betacyanin and betaxanthin content of *Portulaca* sp. cv. Jewel cell cultures drastically increased under continuous illumination, particularly with blue light irradiation along with the increasing number of growth cycles**.**

In addition to blue light effect, continuous white light and 16 hr per day white light were also able to induce betalain pigments. Therefore, while blue light is essential for efficient induction of betalain pigmentation, both 16 hr per day white light and continuous white light might also be involved as minor factors in betalain biosynthesis in bougainvillea callus. From the above findings it can be assumed that continuous light, especially blue light, may stimulate the genes related to betalain synthesis more rapidly than that of other light conditions. Thus, the betalain pigmentation in bougainvillea callus could be convenient system for further investigation on blue light effects.

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