Indian Journal of Agricultural Sciences **84** (8): 957–61, August 2014/Article
https://doi.org/10.56093/ijas.v84i8.43085

Influence of different carbon sources on *in vitro* induction of anthocyanin pigments in callus cultures of petunia (*Petunia hybrida*)

USHA[1], T JANAKIRAM[2], K V PRASAD[3], CHARANJIT KAUR[4], P SRINIVASAN[5] and C TARA SATYAVATHI[6]

Indian Agricultural Research Institute, New Delhi 110 012

Received: 9 January 2014; Revised accepted: 3 June 2014

ABSTRACT

Anthocyanins are naturally occurring compounds that impart color to fruits, flowers, vegetables, and plants. They are probably the most important group of visible plant pigments besides chlorophyll pigments. Apart from imparting color to plants, anthocyanins also have an array of health-promoting benefits, as they can protect against a variety of free radicals through a various number of mechanisms. Development of an efficient tissue culture system for commercial production of anthocyanins requires an integrated approach through manipulation of various media constituents. The effect of varied concentrations of different carbon sources on anthocyanin production in callus cultures of *Petunia hybrida* cv Bravo Blue was studied. Explants from young leaves were cultured on Murashige and Skoog (MS) medium supplemented with MS + IBA (19.6 µM) + Kin. (4.65 µM) + AdS (81.45 mM), 3% sucrose and 0.7% agar. Among the various carbon sources tested, incorporation of Glucose at 5% was found to have earliest pigment induction with maximum response coefficient with highest pigment content (1.36 ± 0.012 CV/g FCW). Highest gain in fresh cell weight was noticed with the addition of sucrose 5% (3.96 ± 0.06 g). When MS medium was supplemented with different concentrations of Galactose, the explants failed to respond.

Key words: Anthocyanin, Callus cultures, Colour value index, Fructose, Galactose, Glucose, *Petunia hybrida,* Sucrose

Anthocyanins are part of plant-derived flavonoid compounds that are responsible for the development of colours ranging from red to blue in different plant parts. Today, interest in anthocyanin pigments has intensified because they can be used not only as food and beverage additives to obtain attractive natural red colouration but also as nutraceuticals to treat a number of human ailments. Considering the high economical and pharmacological importance of secondary metabolites, industries are deeply interested in utilizing plant tissue culture technology for large scale production of these metabolites (Misawa M 1994).

Though the field crop production is advantageous due to the relative ease of cultivation, yet has the disadvantages of variations in availability due to seasonal growth, heterogeneity of cell types reducing the purity and increasing purification costs, and yield losses from disease and predation (Dornenburg and Knorr 1996). These considerations have stimulated the interest in anthocyanin-producing *in vitro* plant cell/tissue culture-based approaches (Zhang and Furusaki 1999). Bio-processing in plant cell culture is attracting much attention because products can still be considered natural, and production quantity and quality is regulated through a continuous, controlled production process. The expression of many secondary metabolite pathways is easily altered by external factors such as nutrient level and growth regulators. Therefore manipulation of the culture environment must be effective in increasing the product accumulation. It will be interesting to investigate the role of sugars or carbon source treatments in accumulation of healthy promotion compounds such as anthocyanins in petunia (*Petunia hybrida*) calli. The role of sugars in promoting pigment accumulation in cultured cells and tissues has been well documented (Vitrac *et al*. 2000).

Beside their role as energy source and structural components, sugars affect cyto-differentiation via their influence as a cytoplasmic and/or vacuolar osmoticum in the cells besides they act as physiological signals in the global expression of pathway genes (Lila 2004). The osmotic stress created by sucrose and other sugars was found to regulate anthocyanin production in many callus and cell suspension cultures.

Part of the Ph D thesis of the first author submitted to the Indian Agricultural Research Institute, New Delhi 110 012.

[1] Ph D Student (e mail: usha17iari@gmail.com), [2] Head (e mail: tolety07@gmail.com), [3] Senior Scientist (e mail: kvprasad@gmail.com), Division of Floriculture and Landscaping; [4] Principal Scientist, Division of Post Harvest Technology; [5] Professor (e mail: srinivasan53@ gmail.com), Division of Molecular biology and Biotechnology; [6] Principal Scientist (e mail: csatyavathi@iari.res.in) Division of Genetics

Sugar types also affect anthocyanin accumulation. In the culture of *Prunus persica*, sucrose was found to be more effective as carbon source than glucose, fructose or starch under a given nitrogen level (Cordts *et al*. 1987). In strawberry cell cultures, anthocyanin accumulation and cell growth were enhanced by glucose, sucrose and fructose (Mori and Sakurai 1994). A close correlation has been found between anthocyanin accumulation and the level of intracellular hexoses (glucose and fructose) in *V. vinifera* cell cultures (Vitrac *et al*. 2000). Jang *et al*. (1997) suggest that anthocyanin biosynthesis is stimulated by the presence of high levels of hexose sugars, mediated through the activity of hexokinase as a sugar sensor and not through the role of these sugars as a general carbon source. It is possible that this mechanism of anthocyanin enhancement may be regulated through the sugar-dependent expression of CHS (Chalcone synthase) as reported by Tsukaya *et al*. (1991).

Elevated carbon sources can enhance anthocyanin production through increased energy supply and/or increased stress via elevated osmotic pressure. Rajendran *et al*. (1992) subjected cultures to high concentrations of both sucrose and mannitol and showed that the resultant increase in anthocyanin production was the result of increased osmotic pressure rather than an enhanced energy supply. Both Nagarajan *et al*. (1989) and Zwayyed *et al*. (1991) observed that anthocyanin accumulation in carrot cells occurred during the growth phase and stopped when cell division ceased, indicating that anthocyanin production was growth associated. Cultures under stress, either from nutrient starvation or osmotic pressure, produce more anthocyanins. It is possible that this is due to the increased availability of precursors for secondary metabolism, arising from reduced primary metabolic activity (Rajendran *et al*. 1992).

Sucrose concentration also proved to be an important factor for modulating anthocyanin accumulation in calluses of *Cleome rosea*. Supplementation of the culture medium with 70 g/L sucrose increased anthocyanin production and supported a high biomass accumulation. However, a decrease of callus biomass was observed on media containing 90 g/L sucrose, probably caused by inhibition of nutrient uptake due to an increase in the osmotic potential of the medium (Simoes *et al*. 2009). Sucrose was found to modulate polyphenol accumulation in *V. vinifera* cell cultures. Indeed, the accumulation of anthocyanins was strongly increased (12-fold) by sucrose (0.15 M) (Larronde *et al*. 1998).

MATERIALS AND METHODS

The study was conducted on *Petunia hybrida* cv Bravo Blue (Solanaceae) plants. Healthy and disease free explants from young leaves were cultured on Murashige and Skoog (MS) medium supplemented with IBA (19.6 µM) + Kin. (4.65 µM) + AdS (81.45 mM), ± 3% sucrose and 0.7% agar. The initial pH of the medium was adjusted to 5.8 using HCl/KOH, before autoclaving. The explants were maintained at 24 ± 1ºC in the dark. After 20-25 days of culture, the callus was isolated from the explants and sub-cultured every 25 days.

To investigate the effect of carbon source addition on anthocyanin production from *Petunia hybrida* calli, the MS media supplemented with different carbon sources with the concentrations of 0, 5, 6 and 7% were prepared and the calli were cultivated. Glucose, fructose, sucrose and galactose were purchased from Sisco research laboratory, Andheri (E) Mumbai was used as elicitor as a medium supplement.

The anthocyanin biosynthesis in callus cultures 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 calli, after 15-day of cultures. The Colour value (CV) index which is an indicator of total anthocyanin was calculated for pigment content and pigment production with the following equations:

Pigment content (PC, CV/g FCW) = $0.1 \times OD_{525} \times$ dilution factor;

Pigment production (PP, CV/test-tube) = pigment content × respective mean fresh cell mass obtained at the end of each culture.

For microscopic study, fresh tissue of pigmented and non-pigmented calli were mounted on glass slides with a cover slip after cleaning thoroughly and viewed with a stereo microscope (Carl Zeiss Discovery.v8, Carl Zeiss MicroImaging, Germany). Digital images were captured with a digital camera (Carl Zeiss Axiovision, software version: Axiovision 4.8.2).

The experiment was laid out in completely randomized design with three independent determinates for each treatment. Data are presented as mean ± standard error and were analyzed using one-way analysis of variance (ANOVA). The differences among means were tested by the post hoc test Tukey's honestly significant difference (HSD) in the statistical software SPSS version 17.0 (SPSS Inc., USA). Significance for the test was assumed at $P \leq 0.05$ or $P \leq 0.01$.

RESULTS AND DISCUSSION

Callus induction

Under our experimental conditions, leaf was found to be the most suitable explant**.** It gave maximum callusing frequency (73.33 ± 3.33) in minimum number of days (8.88 ± 0.16) with minimum microbial contamination (19.80 ± 1.57). This may be due to the higher level of endogenous phyto-hormones in leaves. It is well known that *in vitro* culturability is dependent on the endogenous level of phyto-hormones and is enhanced by exogenously applied growth regulators (Mederos and Enriquez 1987).

Best callus induction and multiplication was observed under the treatment combination of IBA + Kin+ AdS {(19.60 + 4.65 µM) + (81.45 mM)} in respect of days to callus initiation, induction coefficient percentage, and growth status of callus. Combination of IBA + Kin+ AdS {(19.60 + 4.65

μM) + (108.6 mM)} was also found good for the maximum gain in fresh- and dry weight with the highest FW/DW ratio (12.651 ± 0.46). When cultures were exposed to different light regimes, earliest callus induction (9.82 ± 0.39 days) and good callusing percentage (96.79 ± 0.77) were found in leaf explants grown in the complete darkness.

Effect of carbon sources on pigment induction

Among all the carbon sources tested, it was observed that except galactose, all others were able to induce anthocyanin pigments in callus cultures of petunia, though they differ in response coefficient, days to pigment induction and pigment intensification, fresh callus weight, pigment content and pigment production among themselves. Among different carbon sources used, Glucose 5% was found to show maximum response coefficient (94.44 ± 5.55%) which does not differ statistically with 7% sucrose (86.31 ± 0.60%) (Table 1). Minimum number of days for pigment induction and intensification was noticed when callus was cultured on MS medium supplemented with 5% glucose (8.61 ± 0.24 and 16.22 ± 0.59 days respectively) and 5% fructose (8.83 ± 0.42 and 14.66 ± 0.25 days respectively). When MS medium was supplemented with different concentrations of Galactose, the explants failed to respond.

With respect to callus biomass accumulation, it was found that highest gain in fresh cell weight was obtained on the addition of sucrose 5% (Fig 1) (3.96 ± 0.06 g). Growth was suppressed in the stressed cultures. It could be due to inhibition of nutrient uptake owing to increase in the osmotic potential or high viscosity of the medium. Although sucrose is used as a nutrient in culture media, it also acts as an

Table 1 Standardization of carbon sources for pigment induction

Treatment		Response coefficient (%)	Days taken for	
			Pigment induction	Pigment intensification
MS + Sucrose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	82.94 ± 1.60^{d}	10.44 ± 0.15^{cde}	18.72 ± 0.20^{de}
	6%	83.49 ± 1.24^{d}	12.05 ± 0.24^{ef}	21.00 ± 0.54^{f}
	7%	86.31 ± 0.60^{d}	9.55 ± 0.49^{bcd}	16.83 ± 0.25^{c}
MS + Fructose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	41.11 ± 4.84^{c}	8.83 ± 0.42^{bc}	14.66 ± 0.25^{b}
	6%	30.80 ± 7.54^{bc}	10.55 ± 0.62^{de}	17.72 ± 0.53^{cd}
	7%	22.22 ± 5.56^{b}	12.44 ± 0.20^{f}	20.00 ± 0.42^{ef}
MS + Glucose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	94.44 ± 5.55^{d}	8.61 ± 0.24^{b}	16.22 ±0.59^{bc}
	6%	85.12 ± 1.79^{d}	12.39 ± 0.39^{f}	19.44 ± 0.36^{ef}
	7%	83.01 ± 1.66^{d}	11.50 ± 0.63^{ef}	18.72 ± 0.34^{de}
MS + Galactose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	6%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	7%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
HSD (P≤0.05)		8.56	0.86	0.85

*MS: Murashige and Skoog medium; Same letters within a column did not differ significantly at 5% level of significance when compared by Tukey's HSD test.

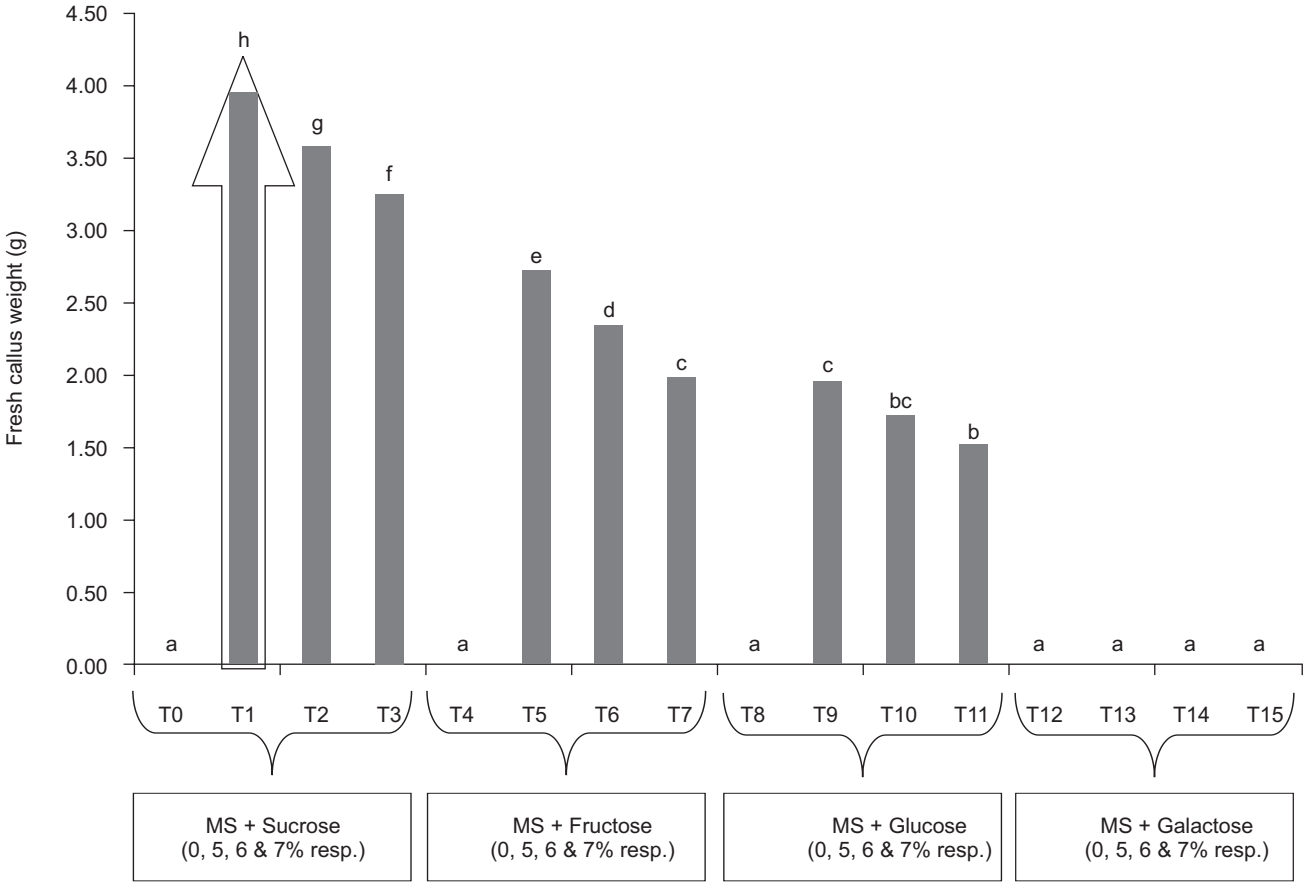

Fig 1 Effect of carbon sources on fresh callus weight of pigmented calli

osmotic agent when present at high concentrations. A dual role of sucrose as carbon source and osmotic agent was also observed in *Solanum melongena* (Mukherjee *et al*. 1991).

Early pigment initiation, enhancement and maximum anthocyanin production from calli were recorded when leaf discs of roses were cultured on *Euphorbia millii* (EM) medium supplemented with 7% sucrose compared with calluses cultured at 4% sucrose concentration under 16/8 h (light/dark) photoperiod regime (Ram *et al*. 2011).

Pigmented callus began to turn brown if maintained on the same medium for more than 4 weeks without sub culturing. This was also observed in anthocyanin-producing calli from *Prunus incise* (S. Zhou *et al*. 2002), *C. rosea* (Simoes *et al*. 2009), *Rosa hybrida* (Ram *et al*. 2012). This could be due to nutrient depletion or oxidative stress due to toxic accumulation of spent materials in the culture medium or secondary metabolites synthesized by stressed cells.

Effect of carbon sources on colour value index (PC and PP)

The percentage of the pigmented cells varied according to the osmolarity of media (Fig 1, 2). Pigmented cells represent the accumulation of anthocyanin in the callus

Table 2 Effect of carbon sources on Colour Value Index (PC and PP)

Treatment		Pigment content (PC; CV/g FCW)	Pigment production (PP; CV/test tube)
MS + Sucrose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	1.03 ± 0.015^{e}	4.07 ± 0.113^{g}
	6%	1.11 ± 0.010^{f}	3.99 ± 0.146^{g}
	7%	1.25 ± 0.015^{g}	4.07 ± 0.092^{g}
MS + Fructose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	0.78 ± 0.012^{d}	2.12 ± 0.023^{e}
	6%	0.70 ± 0.009^{c}	1.65 ± 0.008^{cd}
	7%	0.62 ± 0.012^{b}	1.23 ± 0.072^{b}
MS + Glucose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	1.36 ± 0.012^{h}	2.66 ± 0.105^{f}
	6%	1.13 ± 0.024^{f}	1.95 ± 0.022^{de}
	7%	1.06 ± 0.012^{e}	1.61 ± 0.003^{c}
MS + Galactose	0%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	5%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	6%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
	7%	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
HSD (P≤0.05)	0.06	0.53	

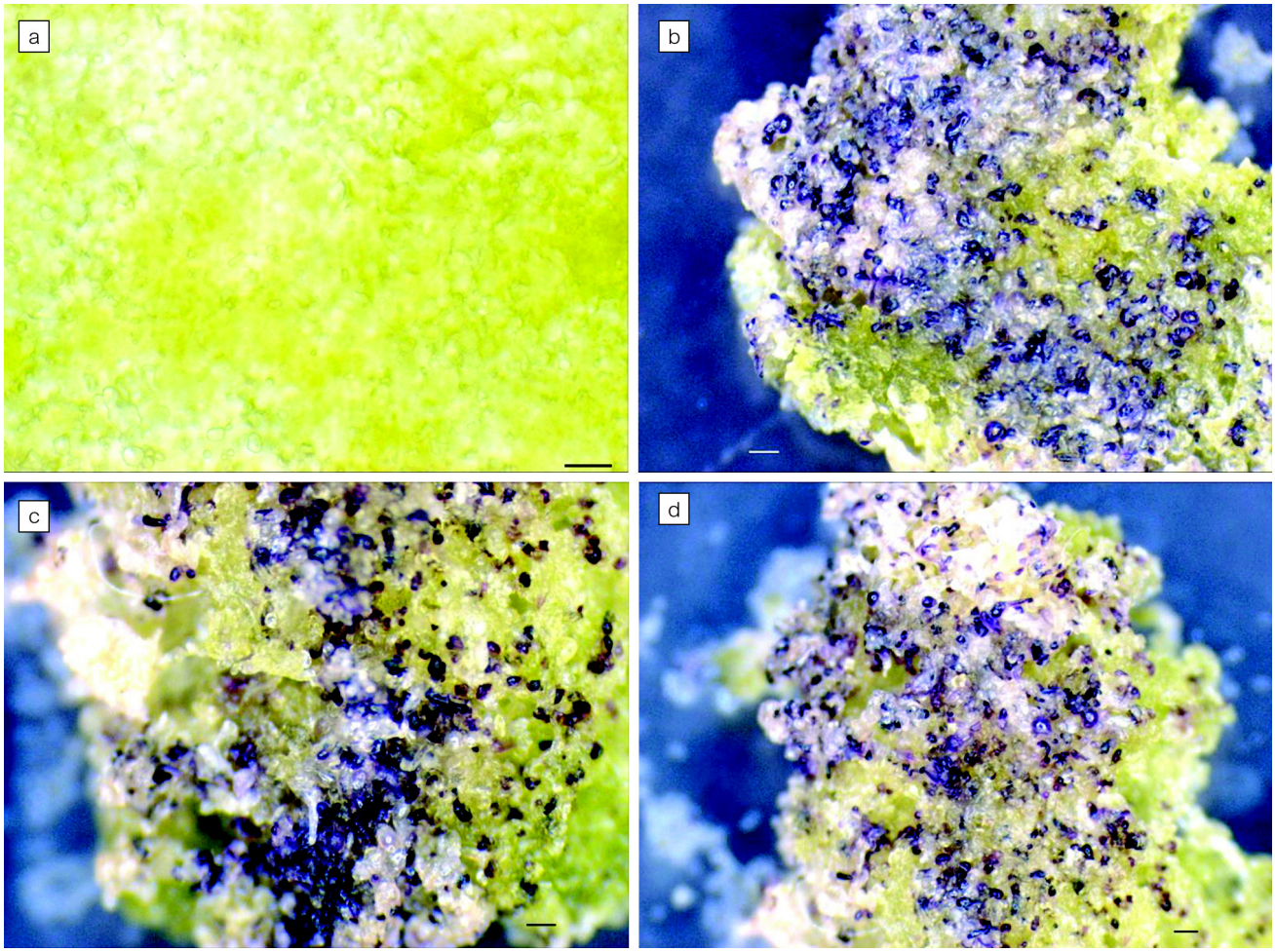

Fig 2 Effect of Glucose on anthocyanin induction. The calli were treated with different concentrations of Fructose 0% (a), 5% (b), 6% (c) and 7% (d) respectively.

cultures of petunia. From the results of colour value index (CV) which is an indicator of total anthocyanin, it was observed that addition of 5% glucose (Fig 2) to MS medium gave maximum pigment content (1.36 ± 0.012 CV/g FCW) which was followed by 7% sucrose (1.25 ± 0.015 CV/g FCW). When pigment production was taken into consideration, maximum response was observed with the supplementation of 7% sucrose (4.07 ± 0.092 CV/test tube) which gave statistically similar results to 5% sucrose supplementation (4.07 ± 0.113 CV/test tube) (Table 2).

Synthesis of anthocyanin in cells might decrease above a level, which might be due to an unusually high osmotic stress (Masahiko S 1995). Unusually high osmotic stress such as in 6% and 7% glucose and fructose might disturb the metabolism of cells and result in the decrease of anthocyanin synthesis. However, no anthocyanin biosynthesis was observed when calli was cultured on media devoid of sucrose (control) and galactose treatments. Do and Cormier (1990) have also reported an increase in the percentage of pigmented cells in *Vitis vinifera* L. in high osmotic medium.

Earlier efforts were focused on developing alternative methods to improve anthocyanin biosynthesis under *in vitro* conditions. The system described here represents a suitable and reliable approach for pigment production in callus cultures of *Petunia hybrida*. The carbon sources affected both growth and anthocyanin production. All the carbon sources tested under our experimental conditions could induce the accumulation of anthocyanins, and glucose and sucrose were the most effective one. Glucose 5% was found to have earliest pigment induction with maximum response coefficient with highest pigment content (1.36 ± 0.012 CV/g FCW). When MS medium was supplemented with different concentrations of galactose, callus cultures failed to respond. When cells were cultured under osmotic stress, total anthocyanin accumulation increased.

In most *in vitro* culture systems, biomass accumulation and secondary metabolite production require different media conditions to induce a shift from the growth state to the metabolite production state, thereby limiting the efficiency of these systems to be used commercially. Therefore, the success obtained with callus cultures of *P. hybrida*, where anthocyanin production was associated with good growth rates on the same medium, makes this protocol a suitable and reliable system for *in vitro* anthocyanin production.

It s very interesting to note that the pigments induced *in vitro* resemble the pigments noticed in the petals of field grown petunia plants of cv Bravo Blue which supports our premise that targeted production of very specific pigments is possible in solid cultures of petunia leaf explants without any seasonal barriers.

REFERENCES

Cordts J, Scorza R and Bell R L. 1987. Effects of carbohydrates and nitrogen on the development of anthocyanins of a red leaf peach (*Prunus persica* L. Batsch) *in vitro*. *Plant Cell Tissue Organ Culture* **9**: 103–10.

Do C B and Cormie F. 1990. Accumulation of anthocyanins enhanced by a high osmotic potential in grape *(Vitis vinifera* L.) cell suspensions. *Plant Cell Reports* **9**: 143–6.

Dornenburg H and Knorr D. 1996. Generation of colors and flavors in plant cell and tissue cultures. *Critical Reviews in Plant Sciences* **15**: 141–68.

Jang J C, Leon P, Zhou L and Sheen J. 1997. Hexokinase as sugar sensor in higher plants. *Plant Cell* **9**: 5–19.

Larronde F, Krisa S, Decendit A, CheÁ ze C, De eux G and MeÂrillon J M. 1998. *Plant Cell Reports* 17: 946.

Lila M A. 2004. Anthocyanins and human health: an *in vitro* investigative approach. *Journal of Biomed Biotechnol* **5**: 306–13.

Mederos S and Enriquez M J. 1987. *In vitro* propagation of golden times roses, factors affecting shoot tips and axillary bud growth and morphogenesis. *Acta Horticulturae* **212**: 619–24.

Misawa M. 1994. Plant Tissue Culture: an alternative for production of useful metabolites. Agriculture Services Bull., FAO, Rome, p 87.

Mori T and Sakurai M. 1994. Production of anthocyanin from strawberry cell suspension cultures; effects of sugar and nitrogen. *Journal of Food Science* **59**: 588–93.

Nagarajan R P, Keshavarz E and Gerson D F. 1989. Optimization of anthocyanin yield in a mutated carrot cell line (*Daucus carota*) and its implications in large scale production. *J. Ferm. Bioeng* **68**: 102–6.

Rajendran L, Ravishankar G A, Venkataraman L V and Pratibha K R. 1992. Anthocyanin production in callus cultures of *Daucus carota* as influenced by nutrient stress and osmoticum. *Biotechnology Letters* **14**: 707–12.

Ram M. 2012. 'Induction and characterization of anthocyanin pigments from callus cultures of rose (*Rosa hybrida* L.).' Ph D thesis, Indian Agricultural Research Institute, New Delhi.

Ram M, Prasad K V, Kaur C, Singh S K, Arora A and Kumar S. 2011. Induction of anthocyanin pigments in callus cultures of *Rosa hybrida* L. in response to sucrose and ammoniacal nitrogen levels. *Plant Cell Tissue Organ Culture* **104**: 171–9. doi: 10.1007/s11240-010-9814-5

Simoes C, Bizarri C H B, da Silva Cordeiro L, de Castro T C, Coutada L C M, da Silva A G R, Albarello N and Mansur E. 2009. Anthocyanin production in callus cultures of *Cleome rosea*: Modulation by culture conditions and characterization of pigments by means of HPLC-DAD/ESIMS. *Plant Physiology and Biochemistry*, **47:** 895–903.

Suzuki M. 1995. Enhancement of anthocyanin accumulation by high osmotic stress and low ph in grape cells *(Vitis* hybrids). *Journal of Plant Physiology* **147**: 152–5.

Tsukaya H, Ohshima T, Naito S, Chino M, and Komeda Y. 1991. Sugar-dependent expression of the CHS-A gene for chalcone synthase from *Petunia* in transgenic *Arabidopsis*. *Plant Physiology* **97**: 1 414–21.

X Vitrac, F Larronde, S Krisa, A Decendit, G Deffieux and J M Me Rillon. 2000. Sugar sensing and Ca2þ-calmodulin requirement in *Vitis vinifera* cells producing anthocyanins. *Phytochemistry* **53**: 659–65.

Zhang W and Furusaki S. 1999. Production of anthocyanins by plant cell cultures. *Biotechnology and Bioprocess Engineering* **4:** 231–52.

Zhou S, Sauve R J and Howard E F. 2002. Identification of a cell wall peroxidase in red calli of *Prunus incisa* Thunb. *Plant Cell Report* **21**: 380–4.

Zwayyed S K, Frazier G C and Dougall D K. 1991. Growth and anthocyanin accumulation in carrot cell suspension cultures growing on fructose, glucose, or their mixtures. *Bio-technology Progress* **7**: 288–90.