Abstract

Anthocyanins are one of the most important and largest subgroup of plant flavonoids. These are water soluble pigments and ubiquitous in higher taxa of plant kingdom, with exception of 10 out of 12 families of the order Caryophyllales (along with some cacti); the betalain producers. The accumulation of anthocyanins in leaves of higher plants is a seasonal phenomenon (autumn colours). These are of widespread occurrence in plant tissues and organs, to be found in all cell types from epidermal to internal vascular tissues; from roots to axillary buds and leaves; from basic bryophytes to advanced angiosperms, in vegetative and reproductive organs. These pigments are responsible for major fruits, flower and vegetable colorations, increasing the aesthetic value along with various medicinal and health benefits. Anthocyanins are popular natural replacement of food colours with a wide range, from pink to magenta-red and purple to stark blue, either for direct consumption or as additives.

Secondary metabolite production is a strategic response of plants to cope with the stressful environmental conditions. By mimicking the stressful conditions in isolated plant cell and culture systems, it is possible to induce the secondary metabolite production. Various strategies have been used for this purpose, out of which elicitation via biotic and abiotic external factors have been extensively and successfully used for secondary metabolite production. Over the past few decades, great emphasis has been directed towards natural and sustainable availability of anthocyanin pigments, which opens new questions and applications in the area of flavonoid research. The anthocyanin biosynthesis pathway is one of the most studied secondary metabolic pathway with a large number of gene sequences available for the regulatory and functional genes for various plant species with a remarkable variation in regulation and behaviour according to the plant species and also the specific accumulating tissues and organs.

Salix tetrasperma Roxb. (Indian Willow) is a widely distributed and easily available energy crop, which proved to be a potential option for in vitro anthocyanin production. The present investigation sheds light on anthocyanin production in response to various external elicitor (BA, CdCl₂, SA & L-Phe) from callus cultures of S. tetrasperma Roxb., derived from two explant sources, viz. leaf and inflorescence. S. tetrasperma is a novel and unconventional anthocyanin producer. The anthocyanin
production and accumulation response differs in different species and different tissues and require a unique set of optimum treatments.

There are very few reports available on successful callus induction in genus *Salix* L. In most instances, the utilization of tissues from vegetative and reproductive origin both, were studied and compared for their callus induction and successful proliferation capabilities. Successful callus cultures were obtained from two explants viz. leaf and inflorescence of *S. tetrasperma*, which appears to be the first report on callus culture induction in this species. Auxins play a significant role in callus induction in most of the plant cultures whether singly or in combination with other plant growth regulator. Of the two auxins tested, viz. 2,4-D and 2,4,5-T, better callus induction efficiency was obtained in 2,4-D treated cultures. Among various concentrations of 2,4-D and 2,4,5-T tested, 2,4-D (2.5 µM) recorded optimum callus proliferation in leaf induced callus while 2,4-D (1.0 µM) was found to be optimum for callus proliferation in inflorescence induced callus.

2,4-D application observed to be beneficial for callus biomass production as well as anthocyanin accumulation. Out of the two explants tested, leaf induced callus was found to be better candidate for *in vitro* anthocyanin production with higher pigment yield over inflorescence induced calli. The combination between plant tissue culture and elicitation strategies is the most promising approach for the production and enhancement of various important secondary metabolites. In anthocyanin production studies *in vitro*, different external elicitation treatments were utilised successfully and exhibited high efficiency for enhancement of pigment production from various plant species.

Most of the plant species have the ability to produce anthocyanins in various organs and tissues. The natural anthocyanin production is most tissues is a seasonal phenomenon and sometimes a response of external environmental stress. A large number of anthocyanin producing plants and organs have been utilized by researchers for *in vitro* anthocyanin production and enhancement studies using various external elicitation conditions. Cytokinin treatment in the form of BA was found to be most promising and effective external stimulus for anthocyanin production and enhancement of anthocyanin content in leaf induced callus. BA (8 µM) was optimum for highest anthocyanin accumulation (5.246 ± 1.30) µg/g FW after 4 weeks of
Abstract

incubation while in inflorescence induced callus, BA (4 \( \mu \text{M} \)) found to be the best, accumulating highest anthocyanin content \((3.156 \pm 1.38) \mu\text{g/g FW}\). CdCl\(_2\) and SA supplementation showed normal accumulation of anthocyanin with non-significant increase in the content over control.

Precursor feeding is an obvious and rational approach for enhancing/ stimulating the plant secondary metabolites using in vitro cultures. Feeding the metabolic precursor of a certain secondary metabolic pathway in the culture medium increase the yield of secondary product in the end. L-Phenylalanine, as the metabolic precursor of phenylpropanoid pathway have been extensively used in culture systems since the beginning of precursor feeding strategies in plant secondary metabolite research. Precursor feeding as early as L-Phenylalanine step i.e. initiation point for general phenylpropanoid pathway, was found to exert no beneficial effects on anthocyanin production. 4 weeks of incubation period was found to be optimum for maximum pigment recovery, and thereafter a gradual degradation of accumulated anthocyanins was observed.

The quality parameters of anthocyanins i.e. low hue and high intensity were found to be associated with high anthocyanin quantity in all of the experiments conducted. A strong positive correlation was observed between high anthocyanin content and increasing colour intensity values.

Correlation between Glutathione-S-transferase enzyme activity and anthocyanin accumulation in vitro strongly suggested towards physical binding of anthocyanin molecules with active site on the enzymes but this effect appears to be dependent upon specific threshold value of anthocyanin content.

The differential gene expression of major anthocyanin biosynthesis pathway genes i.e. PAL, CHS, F3H, DFR and GST revealed the expected expression pattern. All the genes found to be highly upregulated in high anthocyanin accumulating treatments. PAL, CHS, F3H and DFR were found to be upregulated at the onset of pigment accumulation (2 W incubation period) leading to anthocyanin biosynthesis. Decrease in expression at 4 W was found to be associated with completion of pigment biosynthesis.
GST protein serves as an “escort” for anthocyanin molecule and are essential for compartmentalization of the pigments in vacuole. GST gene expression was highly upregulated at 4 weeks (completion of biosynthesis) in comparison to 2 weeks (onset of pigment production) harvesting period, indicating towards association of GST at the later stages, i.e. subcellular transport in vacuoles after synthesis in cytosol. The subcellular transport of the anthocyanin molecules is an essential step for completion of biosynthesis and accumulation of functional molecules. Several models and theories have been proposed for anthocyanin transport to vacuoles on the important role of GST (Glutathione-S-transferases), either directly or indirectly. Differential gene expression analysis of structural flavonoid/anthocyanin biosynthesis pathway genes and GST gene, provided a better insight on the expression and behavioural pattern of genes under high anthocyanin accumulation conditions.

To conclude, the callus cultures obtained from leaf and inflorescence explants of *S. tetrasperma* can be put forward as a new unconventional source of anthocyanin production. Successful callus culture induction followed by pigment elicitation and enhanced accumulation in this plant has opened up a new range of applications and research modules for *S. tetrasperma* callus lines.