

Plant Anthocyanins: Biosynthesis, Bioactivity and *in vitro* Production from tissue cultures



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Abstract

Anthocyanins are a major class of colorful plant pigments, with the exception of chlorophylls, that have long attracted the attention of chemists and biologists, investigating their biosynthesis patterns, metabolism and physiological roles. Belonging to the group of “flavonoids”, these anthocyanins accumulated in the vacuoles, are mainly responsible for the bright and distinct coloration to fruits, vegetables and flowers. Besides attracting pollinators, this particular class of compounds is often considered as potent “anti-oxidants”, largely impacting human health maintenance. Due to their immense importance as dietary nutraceuticals, enhanced production of these anthocyanins from cell/tissue cultures have been extensively explored since the last 2 decades. This review summarizes the different types of anthocyanins, their basic chemistry and biosynthesis, *in vivo* bioactivities and concludes with collation of major reports regarding *in vitro* production of anthocyanins.

Keywords: Anthocyanin; Flavonoid; Phenylalanine; Cardio protection; Elicitation

Introduction

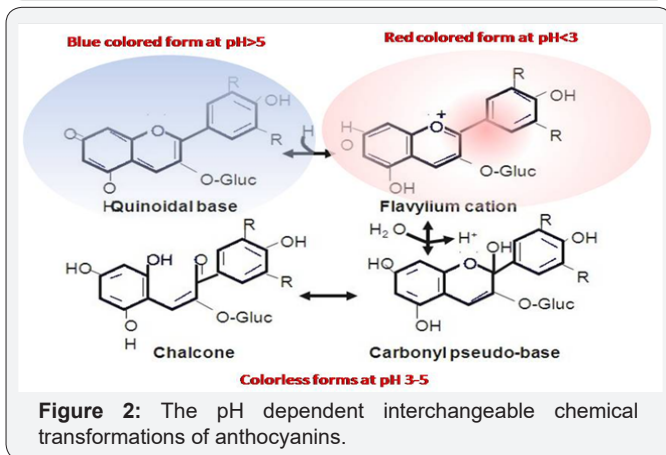
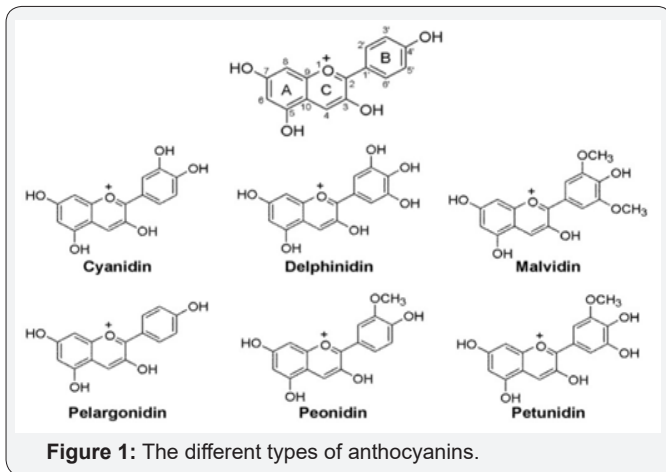
Anthocyanins, one of the most important plant metabolites, are a group of naturally occurring pigments responsible for red-blue coloration in most fruits and vegetables. Belonging to the “flavonoid family”, their structures (more than 500 in number) have been intensively elucidated [1,2]. Anthocyanins are of immense human interest due to their potential implications in maintenance of human health.

These pigments are present in different plant organs such as fruits, flowers and leaves etc [3]. Present exclusively in the vacuoles and for some species in the vacuolar compartment-the anthocyanoplasts, their main sources are red apples, red grapes, berries (blackberry, blueberry, cranberry, raspberry, strawberry), pomegranates, vegetables (red cabbage, red onion, red radish) and purple maize in amounts ranging from 20-1800mg/100g [4]. Flavonoids are a group of secondary metabolites which belong to the class of phenylpropanoids. They are primarily responsible for the red- blue colors found in many flowers, leaves and fruits [5]. Betalains (yellow-to-red) are nitrogen-containing compounds derived from tyrosine. They are also water-soluble and stored in vacuoles, present exclusively in Caryophyllales. Carotenoids are isoprenoids and are found universally in plants and microorganisms, imparting yellow-to-red coloration to flowers and fruits, besides being important

components of plant photosystems. Anthocyanins are believed to be functioning as photo protective pigments for the plant, and preventing oxidative damage. Anthocyanins are found to be induced via stresses such as UV radiation, pathogen attack etc [6].

The Structure and Biosynthesis: The Flavonoid Synthesis Pathway

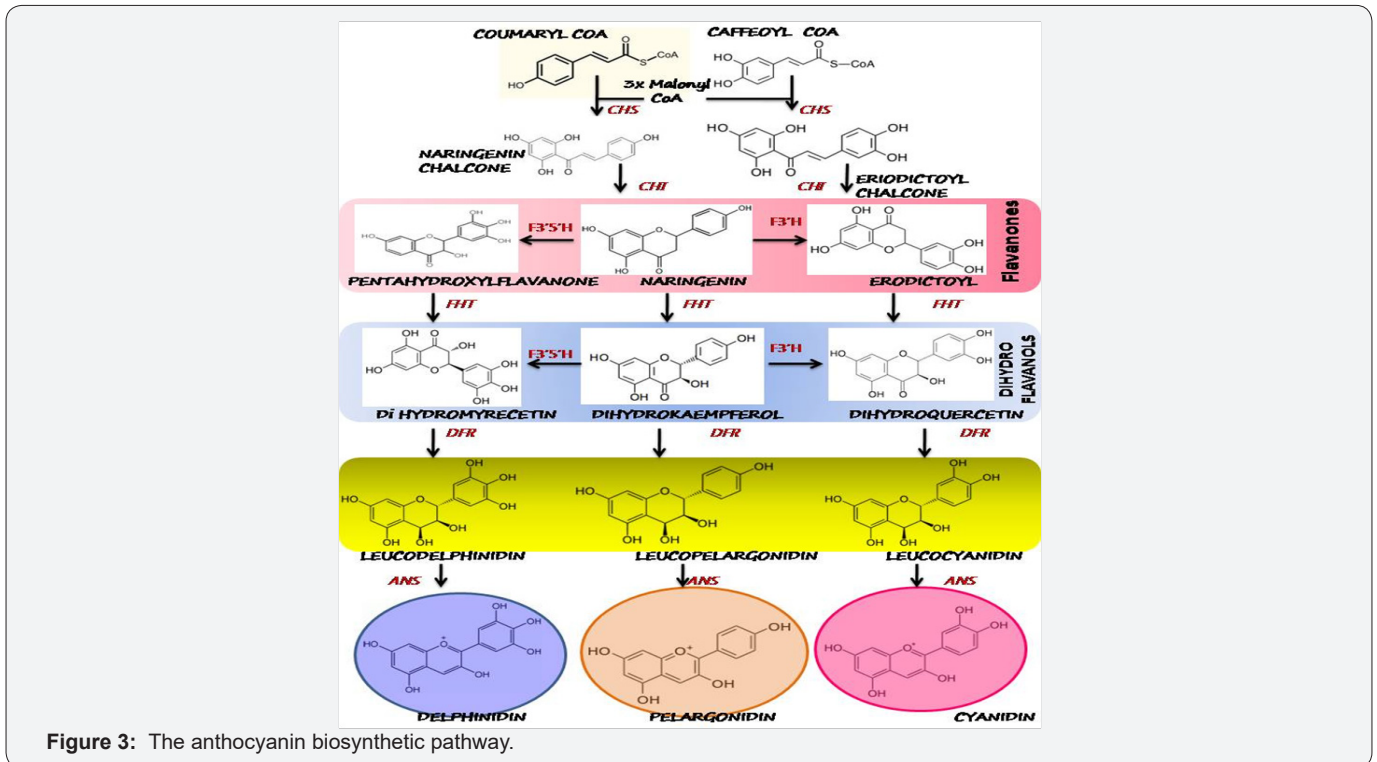
Chemically, anthocyanins primarily possess anaglycone backbone, to which monosaccharides are attached at different positions, resulting in wide variety of flavonoids and colors (pale-yellow to blue) observed in nature. The aglycone forms of anthocyanins are categorized as “anthocyanidins”. A dozen of them have been described, but based on the different hydroxyl/methoxy substitutes, 6 of them are widely present in nature, in fruits and vegetables [7]. The anthocyanidin is typically a flavilium ion (2-phenylbenzopyrylium). The 6 major classes of anthocyanidins are shown in Figure 1 namely cyanidin, pelargonidin, delphinidin and their methylated derivatives malvidin, peonidin and petunidin. These anthocyanidins differ at the hydroxyl/methoxy groups present at the 3' and 5' position. More the number of hydroxyl groups, the bluer the color. More the methoxy group addition, redder the color.



Anthocyanins are extremely water soluble. However, they exhibit a very interesting chemistry in aqueous solutions, with

4 major inter-convertible species, with varying relative amounts at a particular pH. At low pH, flavyliumcation is most prominent with a deep red color. As pH increases, they convert to colorless forms such as pseudobases and chalcones and at a pH more than 5; it changes to a blue colored quinonoidal form Figure 2 [8]. These anthocyanidins are further attached to sugars such as glucose, galactose and rhamnose, via α/β linkage exclusively at position 3 of the aglycon. Alternatively they can also be acylated by cinnamic acid, caffeic, ferulic, malic, oxalic and succinic acid, to name a few [9].

The biosynthetic pathway elucidating the major enzyme systems and chemical transformations are shown in Figure 3. Synthesized cytosolically from phenylalanine, the enzyme systems have been hypothesized to form a “supra-molecular” complex, anchored in the endoplasmic reticulum [10]. Chalcone synthase (CHS) catalyzing formation of chalcones (Naringenin/erodictoylchalcone) from coumaryl CoA or caffeoyl CoA along with 3 molecules of malonylCoA, is the first committed step of the pathway. Subsequently, they are isomerized to yield the typical flavanones naringenin and erodictoyl (Figure 3). Flavonoid 3’ hydroxylase (FHT), is a 2-oxoglutarate-dependent dioxygenase, which catalyzes the formation of dihydroflavanols such as dihydrokaempferol, dihydroquercetin and dihydromyrecitin from the flavanones, naringenin, erodictoyl and pentahydroxyflavanone, respectively. Alternatively as shown in Figure 3, dihydrokaempferol can be hydroxylated at 3’ (F3’H) and 3’-5’ (F3’5’H) to yield the two dihydroflavanols. Flavonoid 3’ hydroxylase and flavonoid 3’5’hydroxylase are CYT P450 enzymes and are necessary for cyanidin and delphinidin synthesis.



(CHS: Chalcone synthase; CHI: Chalcone isomerase; F3'H: Flavanol 3' hydroxylase; F3'5'H: Flavanol 3'5' hydroxylase; DFR: Dihydroflavonol reductase; ANS: Anthocyanidin synthase).

The dihydroflavanols are further catalyzed by dihydroflavonol reductase (DFR), which leads to synthesis of leucoanthocyanidins. In some species of *Petunia*, DFR has been observed to exhibit extreme substrate specificity, rejecting dihydrokaempferol as substrate. They consequently lack the brick-red coloration of pelargonidin in their flowers [8]. Anthocyanin synthase (ANS) again a dioxygenase, finally leads to production of the colored anthocyanidins. These anthocyanidins can further attach a sugar at the 3 position to yield the corresponding glucosides, catalyzed by glycosyltransferases which belongs to the UFGT family (UDP glycosyltransferase).

Many factors seem to operate at regulating anthocyanin accumulation in plants, the most important being environmental conditions such temperature, light intensity etc. Fruits of grape, strawberry and lychee have been reported to show enhanced anthocyanin accumulation by increasing exposures to sunlight [11-13]. Decreased anthocyanin accumulation was observed when grapes were shaded (light intensity decreased) at veraison [14]. Low temperatures have long been known to promote anthocyanin synthesis [15]. Studies in apples have shown that they accumulate more anthocyanin when irrigated with micro sprinkler system at sunset and sunrise [16]. An increased PAL and CHS activity was reported in berries grown at low night temperature [17]. Plant hormones such as ABA [14], ethylene [18] and ethephon [19] have also been shown to increase anthocyanin accumulation. Since it is known that anthocyanin synthesis continues even after harvest, postharvest storage conditions such as maintenance of low temperatures, apt carbon dioxide concentrations, also have a prominent effect on the anthocyanin amount and quality in the food source.

Anthocyanins and Biological Activity

Anthocyanins are primarily antioxidants, exhibit free radical scavenging activity and are reported to manifest a range of bioactivities.

Anthocyanins and cardiovascular protection

Among one of the most studied effects of anthocyanins, they have been observed to have a "heart friendly" tendency. Atherogenesis can be attributed to MCP 1 protein release and anthocyanins have been shown to exert a protective effect against its secretion in human endothelial cells [20]. Similarly anthocyanins have also been shown to prevent release of VEGF (vascular endothelial growth factor), a pro-atherosclerotic factor in vascular cells [21]. In a different study, rats were treated with isoproterenol to induce post infarction remodeling and were fed with red wine which showed a protective effect on hearts by repressing hypertrophy-associated increased phosphorylation of protein kinase C (PKC) α/β II and by activating Akt/protein kinase B (Akt). Anthocyanins also are reported to have an effect

on cholesterol distribution, protecting endothelial cells from CD40-induced proinflammatory signaling. It has been shown that the anthocyanin delphinidin decreases the extent of both necrotic and apoptotic cell death in cultured cardiomyocytes and reduces infarct size after ischemia in rats [4].

Anthocyanins and cancer

Chemo preventive properties have been reported extensively for anthocyanins. Different proteins related to cell cycle and cell death are attractive targets for anthocyanin based cancer prevention. It has been shown that red wine is capable of reducing proliferation of human colon cancer cell line and gastric adeno carcinoma [22,23]. Liu and team also reported prevention of human liver carcinoma cell line proliferation by raspberry extracts [24] the apoptosis inducing effect of anthocyanin glucosides have been reported in leukemia cell lines [25] and hepatoma cell lines [26]. Angiogenesis i.e. blood vessel system development in cancer cells, is a major factor responsible for proliferation of cancer cells. Its inhibition by black raspberry has been found to inhibit tumor development [27]. Even mutagenesis induced by methyl methane sulfonate and benzopyrene has been reported to be significantly inhibited by juices from anthocyanin rich fruits [28]. Further Marko and group (2004) demonstrated the inhibitory effect of anthocyanidins in human vulva carcinoma and colon carcinoma cell proliferation [29]. Clinical trials have shown that the consumption of pomegranate juice can significantly delay the reoccurrence and metastasis of prostate cancer following radical surgery or radiation therapy [30]. Berry (strawberry, raspberry, blackberry, blueberry etc) components, such as anthocyanidins, proanthocyanidins, flavonols, flavanols, stilbenoids, terpenoids, ellagitannins, and ellagic acid target oxidative and UV radiation stress-induced DNA damage and are known to act as chemo preventive agents [31]. Recently it has been reported that consumed blackberries alters innate cell trafficking in esophageal cancer [32]. A decreased expression of the proinflammatory cytokine IL1 β followed with an increased expression of the anti-inflammatory cytokine IL10 was observed. Additionally they also increased the expression of IL12, a cytokine that activates both cytolytic natural killer and CD8+ T cells.

Other bioactivities reported

Anthocyanins have been shown to demonstrate an anti-diabetic effect in rats by Jayaprakasam and group who observed stimulation of insulin secretion under the effect of monoglucosides of cyaniding and pelargonidin [33]. They have also been implicated in protection from hepatic injury [34]. Even ocular defects such as myopic conditions, are reported to improve after anthocyanin administration, however the use of anthocyanins for night vision improvement is still controversial [35]. Anthocyanins have even been reported to exert a beneficial effect on Alzheimer's disease in a transgenic mouse model [36]. They have been reported to demonstrate anti-microbial and anti-oxidative effects [37,38].

In vitro Production of Anthocyanins and their Elicitation

Anthocyanin production has been reported to be biosynthesized *in vitro* in a number of plant systems as elucidated in Table 1 [39-61]. The most extensively studied system is the *Vitis vinifera*, also known as the Cabernet Sauvignon or the humble black grape. Strawberry culture systems have also been investigated for their *in vitro* anthocyanin production potential. The three most important factors that decide the anthocyanin biosynthetic capacity of a particular plant system are sugar concentration, temperature and light irradiation. Light irradiations in *Perilla frutescens* cell suspension culture have been observed to be a positive regulator for anthocyanin biosynthesis with a 1.6g/L anthocyanin yield obtained [38]. In a separate study on methyl jasmonate elicited *Vitis vinifera*, 13.2 fold enhanced anthocyanin accumulation was reported when kept under continuous light irradiation [53]. Maier and team have reported that light irradiation provides stability to two small crucial proteins, Pap 1 and Pap 2, in *Arabidopsis*, which get degraded in dark conditions. Light stabilized Pap 1 and Pap 2 further activate the transcriptional factors that induce anthocyanin pathway structural gene expression [62]. Low temperatures have also been observed to have a stimulatory effect on anthocyanin biosynthesis. However, as observed by [44] biomass accumulation was maximum at 30 °C, after which strawberry cell suspensions were shifted to 20 °C, at which a 4 fold increase in anthocyanin content was observed. Similar phenomenon was also observed with *Perilla frutescens* cell suspensions, with maximum biomass at 28 °C, however with reduced anthocyanin synthesis. Maximum pigment volume could only be attained at 25 °C [42]. The most important and the most extensively studied factor, is the sucrose concentration, which seems to have a significant impact on *in vitro* plant anthocyanin synthesis. Increase in sucrose has been observed to have a direct co-relation with increased anthocyanin accumulation [42,48,52,54,59]. Vitrac et al. [52] conducted a

series of experiments to determine the involvement of calcium and calmodulin in sugar signal perception. They concluded that hexokinase, which phosphorylates the glucose, plays an important role in the sugar sensing. Calcium and calmodulin mediated activation of a cascade of protein kinase/phosphatase activities may help in transferring the sugar signal to the genomic encoded machinery for anthocyanin synthesis [52]. Elicitation strategies have also been employed to enhance anthocyanin biosynthesis, most predominantly in *Vitis*. Abiotic elicitors are frequent as opposed to biotic mode of elicitation. Methyl jasmonate is a fruitful elicitor for *Vitis vinifera* cell suspensions [54,57], with enhancement to the tune of 2.8-4.1 folds. Methyl jasmonate has also been reported to have a positive effect on anthocyanin production in *Rosa hybrida* [51]. They observed that although MeJA had a negative impact on biomass accumulation, but with highest frequency for color response in callus lines (97.25%). Pectins and ABA additions have also been observed to have a stimulatory effect on *Vitis* cell suspensions, in terms of anthocyanin production [56,55]. Precursor feeding mostly phenylalanine has also been reported to have a positive effect on anthocyanin accumulation. Repetitive phenylalanine feeding to strawberry cell suspensions led to an enhanced anthocyanin production, as opposed to cultures which were not fed with the precursor [46]. They observed an 81% increase over the non-fed cultures and a 30% increase over a single fed culture. The authors, however, did not observe any growth inhibition, as is likely with higher doses of phenylalanine. Precursor feeding can also be clubbed with elicitation to modulate the anthocyanin biosynthetic pathway. Qu et al. [57] reported a 3.4 fold increase in anthocyanin yield in *Vitis vinifera* cell suspensions, when treated with 5mg/L phenylalanine and 50mg/L MeJA [57]. Reports on anthocyanin enhancement via biotic elicitors are scanty. Rajendran et al. [40] observed a 27.4% DW anthocyanin content in *Daucus carota* cell suspensions when elicited with mycelial extract of *Aspergillus flavus*. Cai et al. [63] reported a 7 fold increase in resveratrol production in *Vitis* cultures from a biotic elicitor prepared from insect salivae (*Manduca sexta*).

Table 1: Representative examples of *in vitro* anthocyanin production and elicitation studies.

Plant	Elicitor/Manipulation in Nutrient Medium	Anthocyanin Production	References
<i>Daucus carota</i>	None	5.4%DW	[39]
	Elicitation with ME of <i>Aspergillus flavus</i>	27.3% DW	[40]
	MeJA	0.37% DW	[41]
<i>Perilla frutescens</i>	Sucrose 60g/L	>5.8g/L	[42]
	Light irradiation in agitated bioreactor	1.6g/L	[38]
Strawberry	None	120mg/L	[43]
	Temperature (15-30°C)	270mg/L	[44]
	Addition of strawberry conditioned medium	1250µg/FCW	[45]
	L-Phenylalanine feeding	40mg/g dry cell	[46]
<i>Cleome rosa</i>	None	26 CV/g FW	[47]
<i>Camptotheca acuminata</i>	None	350µg/g FW	[48]
<i>Aralia cordata</i>	None	10.7% DW	[49]

<i>Vaccinium pahalae</i>	MeJA elicitation	180mg/L	[50]
<i>Rosa hybrida</i>	MeJA elicitation	97.25% color	[51]
<i>Vitis vinifera</i>	150mM sucrose added at 7 th day	100g/L	[52]
	Light irradiation+ JA	22.62 CV/g-FCW	[53]
	MeJA + Sucrose elicitation	4μmol/g FW	[54]
	ABA Addition	400μg/gFW	[55]
	Pectin elicitation	4mg/g DW	[56]
	MeJA elicitation+ phenylalanine precursor feeding	2.8 fold enhancement	[57]
	Ethephon elicitation	2.2mg/g DW	[58]
<i>Panax sikkimensis</i>	None	7.0%DW	[59]
	None	199mg/L	[60]
	Elicitor+ precursor feeding	3 fold enhancement	[61]

Our team at CSIR-CIMAP has also been extensively involved towards *in vitro* production of anthocyanins since the past 15 years. The team has been working in the field of *Panax* biotechnology especially in unexplored Indian ginseng congeners from the North-East. One such ginseng congener i.e. *Panaxsikkimensis* from Sikkim, India has been explored for its *in vitro* secondary metabolite production. The team has been the first group in India to develop and patent an anthocyanin producing red colored cell line of *Panaxsikkimensis* that could co-accumulate secondary metabolites i.e. ginsenosides as well as anthocyanins [64]. The anthocyanins stably accumulated in this particular cell line were found to be of the “peonidine” type [60]. Cell suspensions developed from this cell line was observed to be a potentially rich source of ginsenosides (77mg/L) and anthocyanins (199mg/L) at the shake-flask level [56]. Studies have been conducted to identify elicitors, combined with precursor feeding strategies that successfully led to 3-4 fold enhancements in productions of anthocyanins from these cell suspensions. These cell suspensions exhibited enhanced biochemical activities of PAL and UFGT enzyme systems in a crude cell free preparation. Alternatively enhanced expression of these genes was also observed when these treatments were subjected to Real Time PCR analysis [61]. Bench level up scaling of these treatments in a 3-5 litre bioreactor is underway.

Conclusion

Anthocyanins as a group of chemical compounds are pretty much well understood in terms of their biosynthesis, enzyme families and regulation. Reports are being constantly generated regarding novel bioactivities, different classes of antioxidants exhibit, *in vivo*. Production of anthocyanins via cell cultures has been investigated since a long time. Efforts to enhance their production have constantly been examined via elicitation and precursor feeding strategies. Effects of physical factors such as light, temperature and postharvest conditions are well defined in terms of anthocyanin accumulation. However, a definitive gap still remains in terms of mechanisms involved in sequestering and vacuolar compartmentalization, cellular trafficking of anthocyanins and their regulation. Answers to these key missing elements will contribute significantly in successful

metabolic pathway engineering/flux diversion for plant pigment biosynthesis. Extensive biochemical characterization of putative “ molecular protein complexes” , determining their structures and use of bioinformatics tools to predict structure-function relationship may also help to explore systems capable of synthesis of novel compounds, difficult to process chemically. Such information may also help in developing biochemical enzyme systems capable of customized plant pigment synthesis with “cellular editing” such as hydroxylation, de-glycosylation etc for desired chemical transformation.

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