Chapter 11

Production of Anthocyanins in Grape Cell Cultures: A Potential Source of Raw Material for Pharmaceutical, Food, and Cosmetic Industries

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Additional information is available at the end of the chapter

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1. Introduction

Research continues to show that many artificial pigments are actually detrimental to our health. According to [1], there is an increasing consumer preference for healthy foods, which has invited considerable demand for the use of anthocyanins as natural colorants, because of their natural pedigree and healthful properties. Anthocyanins are the most widely distributed group of water-soluble plant pigments in nature. They are mainly responsible for the mauve, red, blue, and purple colors in flowers, fruits, leaves, seeds and other organs in most of the flowering plants. The other important class of water-soluble pigments are betalains, which are present only in plants belonging to 13 families of Caryophyllales order [2-5]. An interesting phenomenon is the existence of mutual exclusiveness of anthocyanins and betalains in plant kingdom [3, 5-9]. Recent research demonstrated that simultaneous production of anthocyanins and betalains is possible in cell cultures and seedlings of anthocyanin producing plants by introduction and expression of genes encoding dihydroxyphenylalanine (L-DOPA) dioxygenases in combination with substrate precursor feeding [10]. However, the co-occurrence of both pigments in the same plant species have never been found in nature and the plants which produce anthocyanins never produce betalains and vice versa [6]. The commercial production of anthocyanin pigments is one of the fastest growing segments of the food colorant industry [2, 11]. The only industrial sources for anthocyanin pigments are from whole plant extracts [1], with the most common source being grape skins from the wine industry. According to [1], the demand of natural colorants continues to rise by 5-15% every year and this translated to the sales of anthocyanins isolated from grape skins in 2002, which was estimated to be US$200 million worldwide. The increase

in demand for processed foods and high health products has caused the manufacturers to look for alternative sources of colorants with antioxidant properties. One source is the production of anthocyanins through the use of plant cell cultures [2, 12, 13].

Anthocyanins are synthesized via the flavonoid pathway, and they are known to contribute red, blue and purple color to colored grapes, wines and other products [14-18]. Anthocyanins can be used not only as food and beverage additives to obtain attractive natural coloration [19], but also for generating pharmaceutical and cosmetic products. Most researchers are optimistic about utilizing them as bioactive compounds with the consideration that they have the potential to improve human health [1]. Anthocyanins have been implicated in lowering the risk of cardiovascular disease and certain cancers. Dietary anthocyanins can be obtained by humans through the ingestion of fresh colored fruits processed into food and beverages. For instance, the consumption of red grapes and wine is considered vital for bioavailable anthocyanins [20, 21]. To date, most anthocyanin colorants are extracted from grape skins, black carrots, red cabbage, and sweet potato [11]. However, researchers are also exploring the idea of cultivating plant cell cultures for the production of natural colorants. Therefore, there is an interest in improving the quantity and quality of anthocyanins produced in grape cells, and this means that commercially viable systems must be developed to produce anthocyanins in grape cell cultures.

Production of anthocyanins by plant cell cultures is a feasible technology being pursued by industrial and academic interests. Several strategies are being used to enhance anthocyanin biosynthesis in plant cells. This involves a proper selection of the cell strain and optimization of media as well as culture conditions. It is crucial to note that anthocyanins obtained directly from fresh plant materials has limitations such as low metabolite yield, variability, and seasonal availability of raw materials, fresh material losses, inconsistent product quality, and pigment degradation caused by storage and extraction process [22]. Therefore, it is prudent to use *in vitro* cell and tissue cultures for the production of anthocyanins as the potential alternative to synthetic coloring agents. In order to cultivate plant cell tissues using biological techniques, there needs to be two approaches; 1) cell cultures have to be studied, and 2) clonal propagation techniques have to be developed. According to [23], the study of cell cultures starts when the calli are initiated *in vitro*, for the purpose of finding the optimum media composition that best suits cultivation. It is important to note that during cultivation process, the calli can undergo somaclonal variation as they go through different steps of subculture. However, single lines needs to be screened when the genetic stability is reached, so that the productivity of each cell line can be evaluated with the purpose of using them in cell suspension cultures. In that regard, the production of anthocyanins can be increased in cell suspensions through different ways. The final step is the bioreactor cultivation and scale-up to commercial production of anthocyanins. The last one is a critical step since it is in direct correlation with the economical feasibility of the entire process. In our laboratory, we have more than 10 years experience with *in vitro* cultures of different Native American grape species. We have various types of cell suspensions, obtained from the super-epidermal cells of muscadine berry skins (Noble var.) at two phonological stages: veraison and physiological maturity. The long-term goal of our research is to use these cells for nutraceuticals, cosmeceuticals, and food additive studies.

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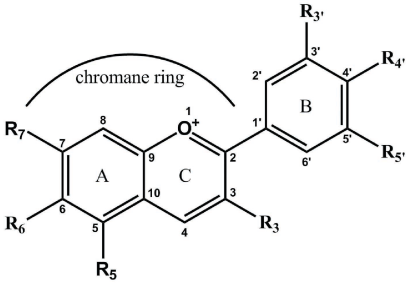
2. Biology and chemistry of anthocyanins as pigments

As colored molecules, anthocyanins play a key role in survival and evolution of flowering plants by attracting pollinators, frugivores and seed dispersers on one hand, and by repelling herbivores and parasites on the other [24-26]. Moreover, anthocyanins execute several important physiological functions in plant cells, and their biosynthesis is strongly induced by biotic and abiotic stress factors. These factors include, light, UV radiation, high or low temperatures, wounding, osmotic stress, nutrient imbalance, ozone exposure, herbivores, microbial and viral attacks. In [24, 27] the major roles of anthocyanins in photoprotection of chloroplasts from photoinhibitory damage have been discussed in details. The authors have also clarified the involvement of anthocyanins in protection from UV-B radiation, as well as how anthocyanins decrease oxidative stress by scavenging free radicals and modulating reactive oxygen signaling cascades. These cascades are responsible for triggering the expression of stress-responsive genes as well as the regulation of plant growth and development [24, 27].

Structurally anthocyanins are substituted glycosides and acylglycosides of 2-phenylbenzopyrilium salts (anthocyanidins). The basic structure of anthocyanidins consist of a chromane ring (C-6 – ring A and C-3 – ring C) bearing a second aromatic ring (C-6 – ring B) in position 2 (Figure 1) [2, 5, 28-30]. The various anthocyanidins differs in number and position of the hydroxyl and /or methyl ether groups attached on 3, 5, 6, 7, 3′, 4′ and/or 5′ positions. Despite the fact that 31 different monomeric anthocyanidins have been identified (including 3-deoxyanthocyanidins, pyranoanthocyanidins and sphagnorubins), 90% of the naturally occurring anthocyanins are based on only six structures (30% on cyanidin **2**, 22% on delphinidin **3**, 18% on pelargonidin **1** and in summary 20% on peonidin **4**, malvidin **6** and petunidin **5**). Those six anthocyanidins are usually known as common anthocyanidins (Figure 1.) [29].



1	Pelargonidin (Pg)	**R3**=OH; **R5**=OH; **R6**=H; **R7**=OH; **R3'**=H; **R4'**=OH; **R5'**=H
2	Cyanidin (Cy)	**R3**=OH; **R5**=OH; **R6**=H; **R7**=OH; **R3'**=OH; **R4'**=OH; **R5'**=H
3	Delphinidin (Dp)	**R3**=OH; **R5**=OH; **R6**=H; **R7**=OH; **R3'**=OH; **R4'**=OH; **R5'**=OH
4	Peonidin (Pn)	**R3**=OH; **R5**=OH; **R6**=H; **R7**=OH; **R3'**=OMe; **R4'**=OH; **R5'**=H
5	Petunidin (Pt)	**R3**=OH; **R5**=OH; **R6**=H; **R7**=OH; **R3'**=OMe; **R4'**=OH; **R5'**=OH
6	Malvidin (Mv)	**R3**=OH; **R5**=OH; **R6**=H; **R7**=OH; **R3'**=OMe; **R4'**=OH; **R5'**=OMe

Figure 1. Structures of common anthocyanidins

The color of anthocyanidins differs with the number of hydroxyl groups, attached on their molecules (especially those substituted in ring B). With the increase of attached hydroxyl groups, the visible color of entire molecule shift from orange to violet (Figure 2) [2, 5, 29, 30]. Glycosylation of anthocyanidins results to additional reddening of obtained anthocyanins, whereas the presence of aliphatic or aromatic acyl moieties causes no color change or slight blue shift and has significant effect on their stability and solubility [5]. Changes in pH can also cause reversible structural transformations in anthocyanins molecules, which has a dramatic effect on their color (Figure 3) [30-34].

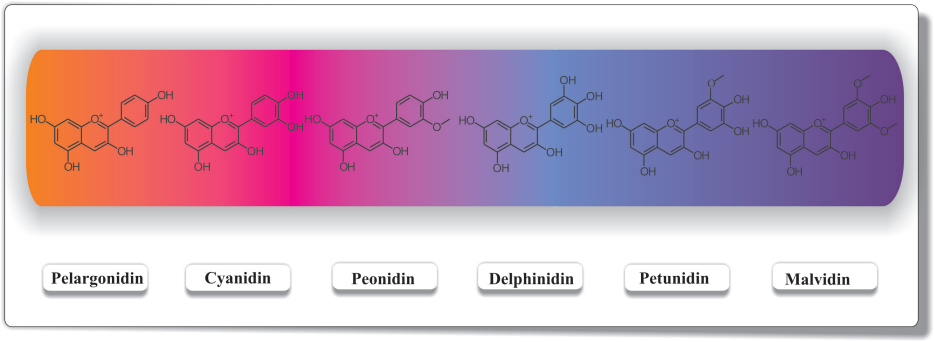


Figure 2. Visible color range of common anthocyanidins

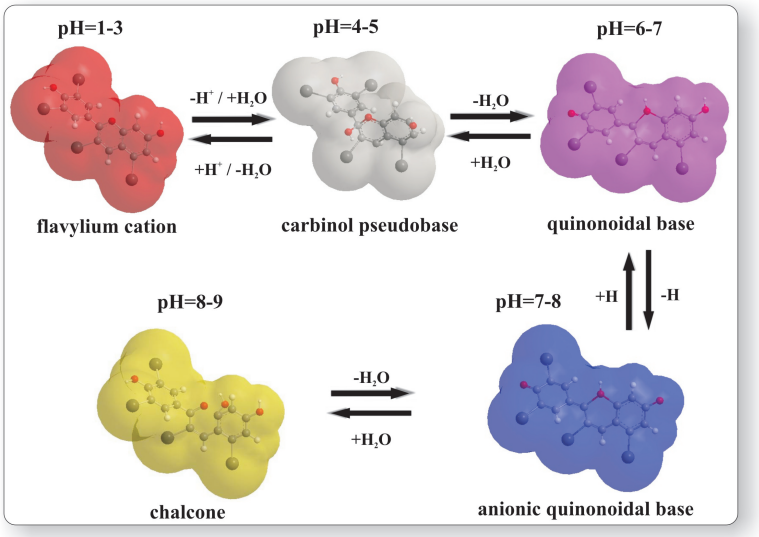


Figure 3. Structural transformations of anthocyanins in aqueous medium with different pH.

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Most of the anthocyanins are *O*-glycosylated at 3 (except those based on 3-deoxyanthocyanidins and sphagnorubinns), 5 or 7 positions and in some cases at 3′, 4′ and 5′ positions [24, 35]. However, 8-*C*-glycosylanthocyanins have been found only in *Tricyrtis formosana* Baker [36, 37]. Anthocyanins contain two, one or tree monosaccharide units in their molecules. The usual monosaccharide residues are glucose, galactose, arabinose, ramnose, xylose and glucuronic acid. However, anthocyanins containing disaccharides and trisaccharides were also found in nature but no tetrasaccharides have been discovered yet [24]. Different anthocyanins based on cyanidin **2** aglycone, found in nature are presented on Figure 4

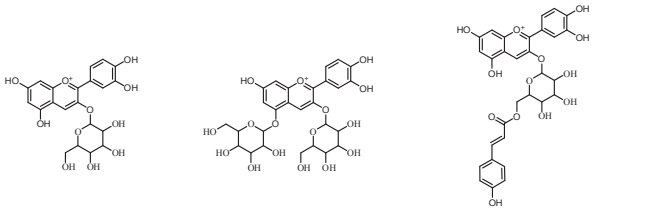

Figure 4. Anthocyanins based on cyanidin aglycone.

2.1. Anthocyanin biosynthesis in grapes

As a major flavonoid group, anthocyanins are products of phenylpropanoid metabolism of plant cells [28, 29, 38]. Anthocyanins in grapes are synthesized via flavonoid pathway. The biosynthetic pathway can be divided into two sections, the basic flavonoid upstream pathway, which includes early biosynthetic genes (EBGs), and the specific anthocyanin downstream branch, which includes late biosynthetic genes (LBGs) (Figure 5). Studies have shown that the basic flavonoid upstream pathway is restricted in many plants [39, 40, 41, 42] and that large gene families encodes the enzymes that act early in the flavonoid pathway, while the enzymes acting late in the pathway are encoded by single active gene [43]. The flavonoid pathway starts with phenylalanine, produced via shikimate pathway and transformed to 4-coumaroyl-CoA. The key enzyme, chalcone synthase (CHS) produce a naringenin chalcone by condense of one molecule 4-coumaroyl-CoA and three malonyl-CoA molecules (derived from citrate produced by The Krebs cycle) (Figure 5) [44]. In this case, the rings A and C are derived from the acetate pathway, whereas the ring B is derived from shikimate pathway [45]. Currently, there are three genes encoding CHS in grapes: *Chs1*(AB015872), *Chs 2* (AB066275), and *Chs 3* (AB066274), which are transcribed under different controls [46, 47]. The three genes act to synthesize naringenin chalcone, which is used in the formation of anthocyanins, proanthocyanidins, and other phenolic compounds. According to [47], the three different CHSs may act in three different pathways to produce different secondary metabolites. In the next step, chalcone isomerase (CHI) converts stereospecifically the naringenin chalcone to its isomer naringenin. Ring B of the naringenin undergoes further hydroxylation by the enzymes flavonoid 3′-hydroxylase (F3′H), flavonoid 3′5′-hydroxylase (F3′5′H) or flavanon 3ß-hydroxylase (F3H) [48]. Then, the obtained dihydroflavonols are reduced by the enzyme dihydroflavonol 4-reductase (DFR) to the corresponding leucoanthocyanidins.

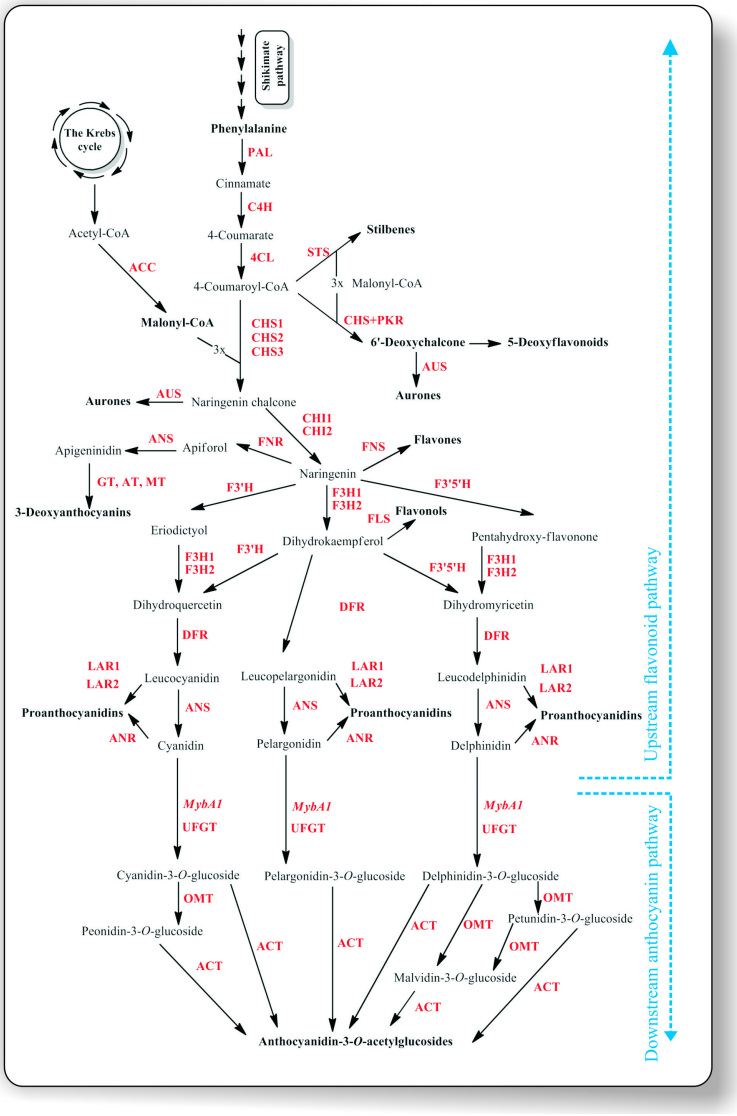

Figure 5. Flavonoids biosynthetic pathways and biosynthetic pathway of anthocyanins in grape: PAL - phenylalanine ammonia-lyase; C4H – cinnamate 4-hydroxylase; 4CL – 4-coumarate:CoA ligase; ACC – acetyl-CoA carboxylase; STS - stilbene synthase; CHS1, CHS2, and CHS3 - chalcone synthase 1, 2, and 3, respectively; PKR – polyketide reductase; AUS – aureusidin synthase; CHI1 and CHI2 - chalcone isomerase 1 and 2, respectively; FNS – flavone synthaes; FNR – flavonone 4-reductase; ANS – anthocyanidin synthase; GT – glucosyltransferases; AT – acyltransferases; MT – methyltransferases; F3'H – flavonoid 3'-hydroxylase; F3'5'H - flavonoid 3'5'-hydroxylase; F3H1 and F3H2 - flavanon 3β-hydroxylase 1 and 2, respectively; FLS – flavonol synthase; DFR – dihydroflavonol 4-reductase; LAR1 and LAR2 - leucoanthocyanidin reductase 1 and 2, respectively; ANR - anthocyanidin reductase; *MybA1* - MYB transcription factor gene [49]; UFGT - UDP-glucose: anthocianidin: flavonoid glucosyltransferase; OMT – *O*-methyltransferase; ACT – anthocyanin acyltransferase.

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After this reduction, anthocyanidin synthase (ANS) oxidize leucoanthocyanidins to their corresponding anthocyanidins. Anthocyanidins are inherently unstable under physiological conditions and were immediately glycosylated to anthocyanins by UDP-glucose: Anthocianidin: Flavonoid glucosyltransferase (UFGT) [48]. Anthocyanins, containing methylated anthocyanidins (peonidin **4**, petunidin **5** and malvidin **6**) as aglycone can be obtained by methylation of hydroxyl groups on the ring B of the cyanidin-3-*O*-glucoside **7**, delphinidin-**3**-*O*-glucoside and petunidin-3-*O*-glucoside by the enzyme *O*-methyltransferase (OMT). Future acylation of produced anthocyanins is possible by the action of different anthocyanin acyltransferases (ACT).

2.2. Anthocyanins storage in grape cells

Once anthocyanins have been produced, they are transported and stored into the cell vacuole. Inside of vacuole, anthocyanins could be connected to specific proteins forming nomembrane intravacuolar bodies, known as anthocyanic vacuolar inclusions (AVI) (Figure 6) [50]. It has been confirmed that AVI plays a critical role in formation of color in flowering plants [50]. Recently, AVI from grape cell suspension were isolated and analyzed [51]. In contradiction with other plants, it was demonstrated that in grape cell suspension AVI consist of complex mix of tannins, anthocyanins (predominantly acylated derivates), proteins and other organic compounds, encased by lipid membrane [32, 51]. It was observed that a strong correlation between the prevalence of AVI structures in grapevine cell suspensions and the increase of their anthocyanin accumulation exist [51]. However, the enhancement of AVI prevalence does affect neither the number of available pigmented cells nor the overall growth rate of suspension cultures. Since AVI plays an important function in the storage and concentration of anthocyanins in cell vacuoles, their perspective role as enhancers of anthocyanin accumulation in grape cell suspensions have been proposed [51].

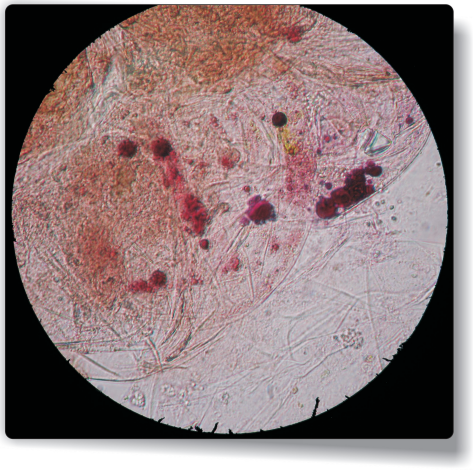

Figure 6. Anthocyanic vacuolar inclusions (AVI) in cell vacuoles of muscadine callus culture.

2.3. Characterization of anthocyanins in grapes

Grapes are rich sources of anthocyanins and bioavailable flavonoids. Grapevines are one of the world's most grown economically important fruit crops. Currently, there are more than 10000 grape cultivars deposited in germplasm collection [52, 53]. Among them, the cultivars of *Vitis vinifera* L. and *Muscadinia rotundifolia* (Michx.) Small., are the most important grapes in Europe and United States, respectively [48]. As a result of crop domestication, numerous changes in grapevine genome have occurred, leading to great variation in berry color [54]. Great differences exist between the vine varieties determined by the presence or absence of anthocyanins in their berries, as well as due to the different anthocyanin compositions of colored berries. It was found that retrotransposon-induced mutations in *Myb*-related *VvmybA1* gene are responsible for appearance of white-fruited *Vitis* varieties [54-56]. The color of grape berries are unique based on the anthocyanin accumulation, and it can be used as "fingerprint" for variety recognition [57]. Thus, *Vitis vinifera* L. varieties produce only anthocyanidin-3-*O*-monoglucosides, anthocyanidin-3-*O*-acetylglucosides and anthocyanidin-3-*O*-*p*-coumarylglucosides, whereas the other *Vitis* species and hybrids produce also anthocyanidin-3,5-*O*-diglucosides [48, 58-61]. Moreover, some *V. vinifera* L. cultivars such as Pinot Noir, red "Chardonnay" and pink "Sultana" produced only nonacylated anthocyanidin-3-*O*-monoglucosides and *M. rotundifolia* (Michx.) Small. accumulates only nonacylated anthocyanidin-3,5-*O*-diglucosides [48, 58, 62-64]. It was demonstrated that the lack of anthocyanidin-3,5-*O*-diglucosides in *V. vinifera* L. varieties is due to occurrence of double mutation in their anthocyanin 5-*O*-glucosyltransferase gene [65]. However, hybrid varieties always produce mixtures of anthocyanidin-3-*O*-monoglucosides and anthocyanidin-3,5-*O*-diglucosides which is used to monitor their usage in winemaking [48, 57]. The aglycones of common anhocyanins found in grapes are malvidin **6**, delphinidin **3**, petunidin **5**, cyanidin **2**, peonidin **4** and pelargonidin **1**. Malvidin **6** is the predominant anthocyanidin in most of the *V. vinifera* L. varieties (with exception of some "Muscat" cultivars in which cyanidin **2** is the major aglycone), whereas pelargonidin-**3**-*O*-glucoside was detected only in trace amounts [48, 66]. Recently trace amounts of pelargonidin-**3**-*O*-glucoside and pelargonidin-3,5-*O*-diglucoside were found to present also in *Vitis labrusca* L. and *Vitis amurensis* Rupr., respectively [57, 67]. The absence of pelargonidin 1 derivates in detectable concentrations in grape could be explained with the higher activities of F3'H and F3'5'H in *Vitis* species, which redirects the metabolite flux to production of cyanidin **2** and delphinidin **3** instead of pelargonidin **1** [48].

2.4. Anthocyanins relevant to berry quality

During their growth, grape berries follow a double sigmoid curve [68]. Veraison is the unique stage of berry development, representing the transition from growth stage to ripening. Once the grape berries enter to veraison, many physiological and biochemical changes occur. The grape cells completely redirect their metabolism to production of secondary metabolites, necessary to prepare berries for reaching the stage of physiological maturity. During this stage, the chlorophyll in berries has been completely lost and the biosynthesis of flavonoids, including anthocyanins is promoted [68]. Microarray analysis showed remarkable overexpression of genes involved in flavonoid biosynthesis and particularly in anthocyanin produc-

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tion during veraison and ripening in *vinifera*, *rotundifolia* and *aestivalis* species [47, 69, 70]. With the progress of anthocyanins accumulation the color of grape berries changes from green through red and then to purple due to the subsequent methylation of produced anthoyanins. This results in the increment of the relative shear of more metabolically evolved anthocyanins in the mature berries [68, 71]. Anthocyanin composition of grape variety is of great importance for estimation of technological properties of berries in winemaking. Thus the varieties, which accumulates predominantly anthocyanins build on anthocyanidins having ortho-positioned hydroxyl groups (cyanidin **2**, delphinidin **3** and petunidin **5**) are sources of more unstable color, compared to varieties, which accumulates anthocyanins based on malvidin **6**, peonidin **4** and pelargonidin **1** [48, 63]. Moreover, the increased amount of acylated anthocyanins significantly contributes to the stabilities of their color [63, 72, 73]. The quantity and quality of anthocyanins plays a crucial role in evaluation of berry qualities and determination of the right time for their collection. To monitor accumulation of pigments in situ, a rapid and non-invasive method based on application of fluorescence sensor has been developed [74, 75]. The method allows anthocyanin content in grape bunches to be monitored non-destructively on the vine in the field and was found to be effective in detecting the earlier ripening processes [74, 75].

3. Transcriptional regulation of anthocyanin biosynthesis in grapes

Anthocyanin biosynthesis in grapes commences only when ripening of the berry begins (termed véraison) and normally continues throughout the ripening phase of growth. Anthocyanin biosynthesis pathway in grapes has been greatly investigated, including intracellular transportation and accumulation [18, 76-78]. Most of the structural genes have been isolated, cloned and characterized, and there is valuable information available on the mechanisms that regulate their expression within the plant cell [29, 44, 79]. Multiple regulatory genes under the complex regulation are responsible for the synthesis of anthocyanin at the transcriptional level [17, 18, 80]. The early biosynthetic genes (EBGs), which are upstream of the anthocyanin biosynthetic pathway, are regulated by several different families of genes called the Myb transcriptional factors, Myc transcriptional factors (encoding basic helix–loop-helix proteins, bHLH) and WD40-like proteins [40-42, 81]. On the other hand, late biosynthetic genes (LBGs), which are downstream leading to anthocyanin formation through glycosylation and subsequent modification (methylation and acylation) are under the specific control of several regulatory factors. Specific regulatory genes have been identified and characterized in *A. thaliana* and they include *PAP1 & PAP2* (Myb family), *GL3 & EGL3* (Myc family) and *TTG1* (WD40 family). These genes are known to regulate the expression of the structural genes involved in the anthocyanin biosynthesis [82-84]. According to [85], some genes belonging to the Myb and Myc factors (e.g MYBL2, MYB4 and BHLH32) also act negatively to regulate biosynthetic pathway of anthocyanins in *A. thaliana*. However, in grapes, R2R3-Myb transcriptional factors have been implicated to control different branches of the phenylpropanoid pathway including anthocyanins, flavonols, and proanthocyanidins. Deluc *et al*. [86, 87], reported that transcriptional factors, *Vvmyb5a* and *Vvmyb5b* belongs to this group. However,

still little is known about the transcriptional regulation of the structural genes involved in anthocyanin biosynthesis throughout berry development.

Recent studies in grapevine indicates that VvMYBA1 and VvMYBA2 transcription factors regulate UFGT gene, which plays a crucial role in the synthesis and accumulation of anthocyanins [56, 88-90], also identified another key R2R3-MYB protein that regulates proanthocyanidins (PA) synthesis in berry skin and seeds. But it is important to note that regulatory genes that control the expression of genes that encodes enzymes located upstream of UFGT have not been identified. It has been suggested that there is a contribution of at least two distinct regulatory complexes involved in the early and late steps of berry development [61]. Another study [86] also revealed that a MYB gene named *VvMYB5a* is associated with the regulation of the flavonoid pathway during the early phase of berry development. The study of transcription factors involved in the later steps of berry ripening is crucial, so that the coordinate regulatory mechanisms of the biosynthetic pathway throughout the berry development can be understood. R2R3-Myb related transcriptional factors such as *VlmybA1-1*, *VlmybA1-2* and *VlmybA2* have been identified as specifically regulating anthocyanin accumulation. Kobayashi et al. [56] and This et al. [54] revealed that, a retrotransposon, *Gret1*, is inserted in the 5'-flanking region of its related non-functional *VvmybA1* gene, and this contributes to the function lose of its transcriptional factor in white *V. vinifera* L. grapes. However, their research indicated that, the *Gret1* insertion is missing from the *VvmybA1* genes in red skinned spots of white cultivars, leaving behind a solo long terminal repeat (LTR), which becomes the functional regulatory gene. This means that red and white spots seen in the skins of grape berries are the result of deletion of the inception region of the retrotransposon, *Gret1* [91]. *VvmybA1* factor is regarded to be the major gene determining the synthesis of anthocyanin in the grape skin, thus the red and white color of the berries [55, 92]. In addition, *VvmybA1* and *VvmybA2* genes have also been reported to belong to the *VvmybA* family regulator genes in grapes, and they are also responsible for the color accumulation of the grape berries [91].

4. Muscadine grape cells as model system

Muscadines (*Muscadinia rotundifolia* (Michx.) Small.) are native grapes of North America and they are considered the most important cultivated grapevine species in the Southeastern part of the United States. Muscadine grapes are well known for their characteristic flavor and popularity in making juices, jellies, jams, and wine. It is important to note that muscadines are the only grapes that contain ellagic acid, which is known for its anticarcinogenic activity. In recent years, their production has increased significantly because of health promoting effects. They are known to have thick, tough skins that result in relatively low yield in juice, and therefore, 40% to 50% of the berry skins, pulp and seeds have not been traditionally used. The utilization of muscadine cell lines could have an important economic influence on the muscadine industry by expanding the uses of muscadine beyond the traditional jellies, jams, juices, and wines. Our laboratory (Viticulture lab at Florida A&M University) has started to investigate the feasibility of using *in vitro* red cell cultures [93] to improve human health. These cells were established from super-epidermal cells of muscadinia 'Noble' var [93]. They have been

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cultivated from a hard calli to fine cells both in suspension and in solid media. Current functional genomic studies suggest that there is an elevated expression of anthocyanin biosynthetic pathway genes in the *in vitro* red cells of muscadine [69, 94]. Other studies are also underway to evaluate their phenolic compounds and transcriptomics. However, it is important to note that gene expression studies of flavonoid biosynthetic genes are crucial for anthocyanin biosynthesis in the red cells of muscadine.

4.1. Genetic modification: A promising strategy to up-regulate or down-regulate the production of anthocyanins in muscadine grape cells

Genetic engineering of plants has lots of benefit in the agricultural field [95-97]. It contributes to an efficient and cost-effective way to produce a wide array of novel, value-added plant and food products in an environmentally friendly manner. Most scientists including Butelli et al. [98] have highlighted the interest of producing crop plants and their products enriched with health-promoting natural compounds. These compounds include anthocyanins and flavonoids, which have become the targets for improving the nutritional value of foods. This requires an in depth knowledge of the molecular mechanisms underlying the biosynthetic pathways of secondary metabolites in plants as demonstrated by [98]. Even though there are some plants that contain high levels of anthocyanins such as blueberries, there are some species where the accumulation of secondary metabolites is not enough. This is why genetic engineering has been used as a strategy to modify flavonoid biosynthesis in order to enhance flower pigmentation in ornamentals and fruit plants [99-103]. Genetic engineering has become increasingly important worldwide because it provides significant improvements in the quantity, quality, and acceptability of the world's food supply and may be the best source for food security [5, 104]. Currently there is an increased production of plant-based products with an enhanced antioxidant capacity, which is facilitated by this technology [103]. An example of this approach is in tomato, which is also an important vegetable crop worldwide [98, 105, 106]. Several transgenic approaches have been used to enhance the accumulation of flavonoid levels in grape berries and tomato fruit by overexpressing either the structural or regulatory genes involved in the biosynthetic pathway [87, 107]. Most of these studies have been carried out *in vivo*, however, none of these have been exploited in the cell models. Although a significant increase in the final content of some flavonoids (flavonols in particular) has been achieved [108-111], the production of anthocyanins in cell cultures still needs to be exploited. Commercial application of grape cell cultures for production of anthocyanin-based colorants is being delayed due to the following main problems: 1.) biosynthetic instabilities of grape cell suspensions; 2.) yield and productivity are too low to justify commercial production economically; 2.) the capacity of cultured cells for anthocyanin accumulation is limited; 3.) anthocyanins are exclusively intracellular and the development of two-phase process based on their continuous secretion and recovery from the medium is difficult; 4.) anthocyanins with desirable application properties may not be achieved using conventional cell line selection and process manipulation. Majority of these problems can be overcome using genetic engineering strategies by focusing on the functional genomics, transcriptomics, and proteomic studies. This will give us the

ability to select a fast growing cell lines with the ability to produce high yield of target anthocyanin types. Some of the most effective genetic engineering approaches for manipulation of flavonoid biosynthesis pathway in grape cell suspension are discussed bellow by presenting our experience with Native American muscadine grape cells.

4.2. Modification of the anthocyanin pathway using regulatory genes

The final assembly of secondary metabolites in plants is determined by the coordinate transcriptional control of structural biosynthetic genes. Based on the information provided in [112], modulation of the rate of initiation of mRNA synthesis depends on the specific transcription factors, which interact with promoter regions of targeted genes. The regulatory genes that control the pattern and intensity of anthocyanin pigmentations through regulating the expression of several flavonoid-anthocyanin structural genes have been identified in many plants [16, 113-115]. There are two families of transcription factors i.e *MYB* and *MYC* that are involved in tissue-specific regulation of the structural genes found in the anthocyanin biosynthesis [14, 116-118]. It is notable that these transcription factors have a sequence homology from different plant species that shows that they have a common ancestor. In our previous study, an ectopic expression of the *MYB* gene in embryonic cell lines of muscadine grapes confirmed that these regulatory genes can be used for genetic modification of cell cultures in order to increase the final anthocyanin accumulation in cells [119]. However, it is vital to note that the quantity and class of anthocyanin produced might depend on several factors including 1.) How the transcription factor binds to the specific promoter site of the targeted structural gene, 2.) The ability of the endogenous transcription factors to cooperate with the introduced regulators, and 3.) How functional the endogenous transcription factors are [8, 116]. It is important to note that the enhancement of anthocyanin production using genetic engineering was achieved more than a decade a go in model plants such as *Arabidopsis*, tobacco, maize, and tomato [109, 120]. In *Arabidopsis* and tobacco, this was done by introducing the maize regulatory genes R and C1 [121]. The over-expression of the regulatory gene R by itself was able to trigger anthocyanin accumulation in the tissues that originally did not produce anthocyanin in *Arabidopsis* and tobacco [121, 122]. But, when the C1 gene was expressed by itself, there was no pigmentation. In another study carried out by Lloyd et al. [121], the over-expression of CI and LC gene in *Arabidopsis* triggered the accumulation of anthocyanins in tissues that normally do not contain them. The same applies to transgenic cherry tomatoes that showed the accumulation of anthocyanins in the leaves, stems, sepals, and veins, when LC gene was over-expressed [122]. Recently, Deluc et al. [87], isolated *VvMYB5b*, a transcription factor that encodes a protein that belongs to the R2R3-MYB family of transcription factors in *V. vinifera* L. This protein displays significant similarities with *VvMYB5a*, another MYB factor that has been shown to regulate flavonoid synthesis in grapevine [86]. Transient expression of the cDNAs for VvMYB5a and VvMYB5b in grape cells confirmed that they can activate the grapevine promoters of several structural genes of the flavonoid pathway [87]. Deluc et al. [87], also determined that the over-expression of VvMYB5b gene in tobacco lead to an up-regulation of genes encoding enzymes of the flavonoid pathway and also triggered the accumulation of anthocyanin and proanthocyanidin compounds.

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In addition to the over-expression of the transcription factors, the suppression or negative regulators of flavonoid biosynthesis have been described [123]. For instance, high pigmented phenotypes (hp-2) in tomato were revealed when Bino et al. [123], mutated the DE-ETIOLATED1 gene (DET1). The fruits produced from these mutants are dark, and it is because of the elevated levels of flavonoids and carotenoids. The suppression of the regulatory gene *Det-1* resulted in increased levels of secondary metabolite groups. Further study of the mutants by Davuluri et al. [124], indicated that flavonoid levels were increased up to 3.5 fold, lycopene content was two-fold higher and *b*-carotene levels accumulated up to ten-fold compared to wild type fruits. This is an indication that we can use both over-expression and suppression (RNAi) techniques to improve anthocyanin production in grape cells.

4.3. Modification of the anthocyanin pathway using structural genes

The regulatory gene families MYB and MYC control the structural genes within the grape anthocyanin biosynthetic pathway [87, 107]. But it is important to note that, the way in which the structural genes are regulated in grape berry skins appears to be different from the patterns observed in snapdragon, petunia, and maize [125-127]. There are two ways in which the pattern of gene expression in grape berry skins could be explained in relation to regulatory genes; 1.) early biosynthetic genes, which induces the expression of all of the structural genes except UFGT, and 2.) late biosynthetic genes that results in the induction of expression of all structural genes [1]. Alternatively, two types of regulatory genes may be present, one that controls expression of PAL, CHS, CHI, F3H, DFR and LDOX and another that induces UFGT gene expression [43]. This means that the regulatory gene that controls expression of PAL, CHS, CHI, F3H, DFR, and LDOX is expressed early in berry development. But it is crucial to note that many studies have identified UFGT, as the major control point to anthocyanin biosynthesis in grape berry skins, and this control is later in the pathway than has been observed in the studies of maize, petunia, and snapdragon anthocyanin biosynthesis.

Irrespective of the function of the regulatory genes, ectopic expression of the structural genes can also enhance the accumulation of anthocyanins. A study carried out by Muir et al. [108] determined that an ectopic expression of the Petunia *CHI* gene in tomato fruits increased total flavonoids up to 70-fold in tomato fruit peel. Particular flavonoids increased consisted mainly of the flavonols rutin (quercetin 3-rutinoside) and isoquercetin (quercetin-3-glucoside), and to a smaller but still substantial extent of kaempferol glycosides. In another separate study, Colliver et al. [128] increased the amount of flavonoids in the flesh of tomato fruit by introducing a four-gene construct that lead to concomitant ectopic expression of structural genes, *CHS, CHI, F3H* and *FLS* in tomato fruit. This resulted in increased levels of flavonols in both peel (primarily quercetin glycosides) and flesh (primarily kaempferol glycosides) [128]. When expressed separately, none of these four genes was sufficient to lead to flavonol production in fruit flesh. This kind of approach can be replicated in grape cell cultures, by concomitantly over-expressing structural genes such as *DFR* and *LDOX* to enhance the accumulation of anthocyanins. These studies indicate that transgenic approaches can be taken to increase anthocyanin production levels in muscadine grape cell cultures by overexpressing either the structural or regulatory genes involved in the biosynthetic pathway.

4.4. Blocking specific steps in the anthocyanin biosynthetic pathway using RNAi: control of the flavonoid flux

Silencing the structural or regulatory genes on the anthocyanin pathway in muscadine requires the following steps: 1) isolation of the endogenous structural or regulatory gene; 2) construction of the transformation cassettes using structural or regulatory-gene fragments as transgenes; 3) transformation of transgenic red cells via *Agrobacterium tumefaciens* and regeneration; 4) molecular analyses to identify transgenic muscadine cell lines; 5) Protein analyses of transgenic cells to confirm the suppression of flavonoid-related proteins.

The flow of genetic information dictates that "DNA is transcribed into RNA that is translated into a protein" (Fig 7A). Flavonoid biosynthetic proteins are produced using this concept. To shift the metabolic flux in muscadine grape cells, one can consider either 1) over-expressing the genes on the flavonoid pathway or 2) to knock out the production of the flavonoid proteins. Blocking the production of flavonoid proteins can be done by interfering with the flow of the genetic information. For example, to eliminate the production of the muscadine DFR protein (Fig 7B), we could interfere either at the mRNA transcription level (transcriptional gene silencing [TGS]) or at the post-transcriptional level (PTGS). The advancements made in genetic engineering have led to the possibility of knocking out the production of specific proteins in organisms by downregulating and/or silencing the genes encoding these proteins. Strategies developed to downregulate genes in plants include mutation-based reverse genetics [129], gene targeting [130], antisense RNA [131], cosuppression [95, 132], and RNA interference (RNAi) [133]. Genetic and biochemical evidences suggest that antisense-mediated gene silencing, co-suppression, and RNAi are all inputs into a common RNA silencing pathway triggered by the formation of a double-stranded RNA (dsRNA). This pathway, called PTGS, is characterized by accumulation of 21 to 25 nucleotides, small-interfering RNAs (siRNAs), sequence-specific degradation of target mRNA, and methylation of target gene sequences [134]. A typical example is demonstrated by Muir et al. [108], where they used RNAi to blocked specific metabolic conversions in the endogenous tomato flavonoid biosynthesis pathway by down-regulating the expression of specific structural flavonoid genes. In another study, Schijlen [135] also used RNAi technique to inhibit tomato *CHS1* gene and this resulted in a strong reduction of total flavonoid levels (naringenin chalcone and quercetin rutinoside). Based on these data, they decided to use an RNAi-mediated gene construct to block the flavonoid pathway leading to flavonols at CHS, F3H and FLS [103]. In all these studies, a clear reduction of flavonols was obtained by introducing an RNAi construct. But it is important to note that when the FLS RNAi-construct was introduced, there was high accumulation of anthocyanins in the vegetative tissues such as stems, leaves, and flower buds as a result of the decreased activity of FLS. Therefore, based on these studies, it is possible that dihydroflavonols as the natural substrates for both FLS and DFR were efficiently converted into anthocyanins [103]. This is because the decrease of FLS activity may have caused less competition between the flavonol and anthocyanin branches in vegetative tissue of FLS RNAi-tomatoes, thereby improving the metabolic flux towards anthocyanin end products. This is a demonstration that RNAi technique can be used to re-direct the metabolic flax in cell cultures to produce more anthocyanins or flavonoids based on the consumer's interest.

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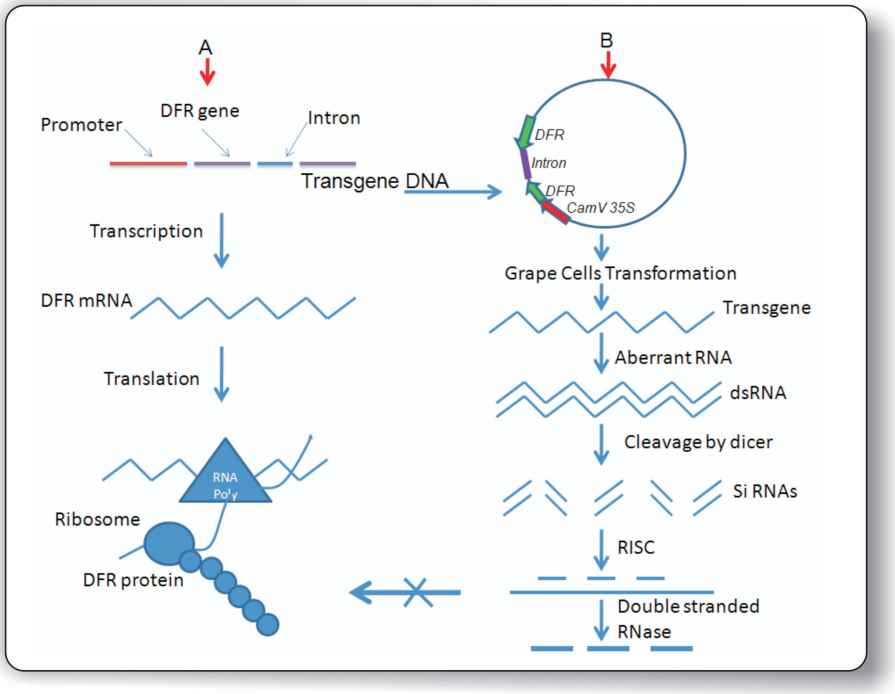

Figure 7. Schematic representation describing the flow of genetic information. **A,** The Central Dogma of Molecular Biology: *dfr* gene is transcribed into a messenger RNA, which is translated into a DFR protein. **B,** Schematic representation highlighting key steps in silencing structural or regulatory genes based on the RNAi model using DFR as an example. dsRNA—double-stranded RNA; RNAi—RNA inteference; RISC—RNA-interfering silencing complex; RNase—ribonuclease. Post-transcriptional gene silencing is initiated by dsRNA molecules that mediate the degradation of homologous transcripts. **B** gives a schematic representation of the molecular process that would be involved in the degradation of mRNA transcripts from muscadine *DFR* genes, based on the PTGS model. A fragment of the *DFR* gene is amplified by polymerase chain reaction (PCR) and cloned into a transformation vector. This vector is a DNA vehicle, which transfers the *DFR* gene fragment (called transgene) into muscadine grape cells following *Agrobacterium*-mediated transformation. The transgene integrates into the endogenous DNA of muscadine cells for expression. Transcripts from the transgene initiate long dsRNA molecules, which would be processed into small 21 to 25 nucleotides (siRNAs) by an endogenous RNAIII enzyme called "Dicer" [136]. These siRNAs would guide the RNA-interfering silencing complex (RISC), which contains the proteins necessary for unwinding the double-stranded siRNAs, and cleave the endogeneous *DFR* mRNAs at the site where the antisense RNAs are bound [137]. Sources of dsRNA formation from *DFR* transgene transcripts include: 1) pairing of transcripts transcribed from an inverted repeat (IR) transgene; 2) pairing of the normal sense RNAs and antisense RNAs (asRNA) arising from aberrant transcription of the transgene; 3) pairing of complementary regions of RNA degradation products; and 4) pairing of transcripts with antisense RNA (asRNA) produced by RNAdirected RNA polymerase (RdRP) [138, 139].

5. Key concepts for enhancing anthocyanin biosynthesis in grape cell lines

Grape cell suspension cultures have been extensively studied as an model for elucidation of anthocyanin biosynthesis pathway, for performing functional genetic studies, somatic embryo

development and most importantly as an alternative source of natural colorants [1, 69, 140-142]. Cultivation of plant cells in controlled conditions offers advantages of continuous supply of high quality anthocyanin pigments. However, scientists have tried for over 40 years to produce anthocyanins in different cell systems, but until now, no commercially feasible anthocyanin producing system has been developed. Switching the production of natural pigments from the traditional approach (involving implementation of numerous agricultural activities) to modern industrial biotech factories is not an easy task. But it is obvious that for the successful realization of such biotechnological advances, research on both empirical and rational levels have to be performed [143]. To succeed in such challenges, we propose to follow a simple integrated approach based on consecutive conduction of various multidisciplinary experiments, optimization and monitoring procedures (Figure 8). As we already discussed some of the rational approaches for manipulation of anthocyanin biosynthesis on genetic level, in the next few pages, the basic principles of empirical studies are highlighted and the current progress on them has been reviewed.

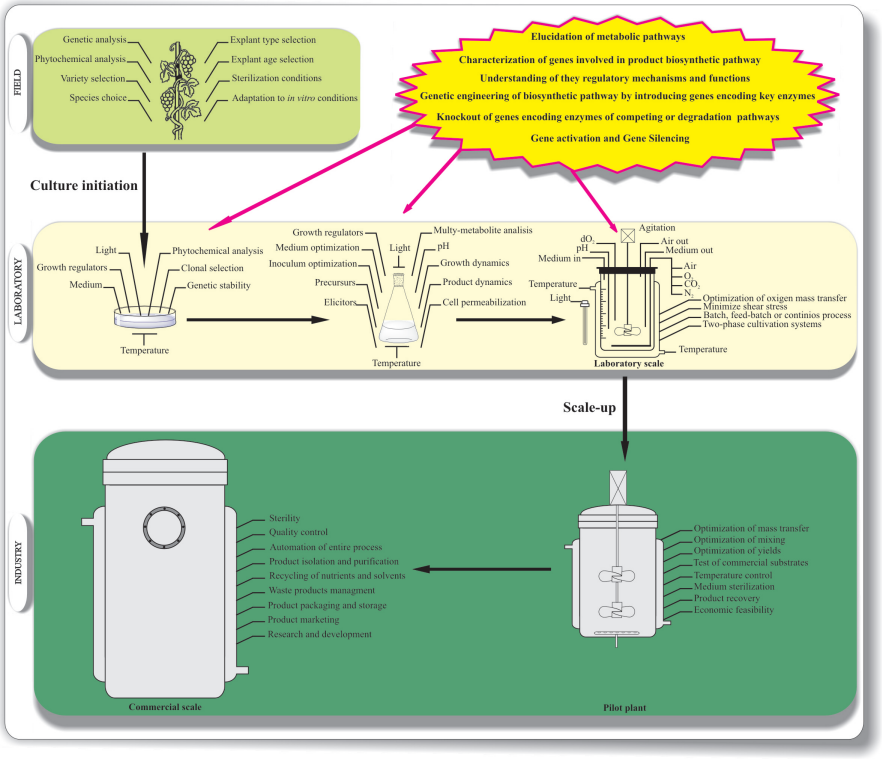

Figure 8. Basic concepts for the development of biotechnological system for anthocyanins production based on grape cell suspension culture. The key steps in bioprocess engineering and optimization of anthocyanins production, involving application of both empirical and rational approaches are presented on each technological stage.

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5.1. Cell line development and improvement

The first step for creation of biotechnology process for anthocyanins production is the development and selection of high producing cell line. To facilitate this process several important preliminary questions needs to be answered. The most important is the right choice of plant species (respectively, the appropriate cultivar) having the necessary anthocyanins profile in both quantity and quality aspects. Recently Lazar and Petolescu [144], generated cell suspension cultures of six grapevine varieties (Burgund Mare, Cabernet Sauvignon, Merlot, Oporto, Negru Tinctorial and Pinot Noir). They cultivated them in a laboratory bioreactor and demonstrated that the growth rates and biosynthetic potential for anthocyanins production were in strong dependence of cultivar used for culture initiation [144]. Currently, most of the research on grape cell suspensions have been performed with cell lines derived from two sources – *V. vinifera* L. *cv*. Gamay Fréaux and *Vitis* hybrid Bailey Alicant A (*V. lincocumii* x *V. labrusca* x *V. vinifera*) x (*V. vinifera* x *V. vinifera*) [1, 48]. Both of them are teinturier cultivars which usually produce malvidin-derivatives in berry skin and peonidin-derivatives in the pulp [145]. Cell suspension cultures of *V. vinifera* L. *cv*. Cabernet Sauvignon (CS4 and CS6) were also reported [146]. In our laboratory we have patented anthocyanin producing cell suspension cultures of *Muscadinia* spp. and *V. aestivalis* var. Cynthiana, which produces mainly anthocyanidin-3,5-*O*-diglucosides [93].

After the choice of targeted variety, the critical step is the selection of appropriate explants, which are used for callus initiation. The type and the age of explants, as well as the environmental conditions at which they are collected are critical factors for successful initiation of *in vitro* cultures [147]. Sterilization procedure, applied to the explants should be as gentle as possible and the necrotic ends of the sterilized cuttings must be removed to avoid the secretion of phenolic compounds into the induction medium. In some cases, the addition of activated charcoal or antioxidant mixture into the culture medium is necessary [147]. Two-phase cultivation systems with adsorbent resin like Amberlite XAD4, which have been recently applied in shoot regeneration and hairy root induction, could be also a prospective alternative for reducing explants browning [148, 149]. One of the most typical characteristic of grapevine callus cultures is their high heterogeneity concerning coloration. The obtained calli usually exist as a mixture of colorless, yellow and red clusters [1, 142, 150]. Therefore, the repeated selection and subcultivation of different-colored clusters can be applied for screening of new high producing lines [142, 146, 150, 151]. As a result of such selection procedure, significant changes in anthocyanin profiles of isolated lines could be observed [142, 146]. Usually those changes lead to the elevation of the 3'-methylated and acylated anthocyanins in grape cell suspension cultures and therefore greatly improve the quality of overall pigments with regards to their color stability [72, 142, 152]. As an example, in cell suspension of *V. vinifera* L. *cv.* Gamay Fréaux, the major anthocyanins were found to be cyanidin-3-*O*-glucoside **7**, peonidin-3-*O*-glucoside and cyanidin-3-*O*-*p*-coumarylglucoside **9** [153]. By repeated selection of colored aggregates from the same culture, Krisa and colleagues obtained a line, which produce remarkably high level of malvidin-3-*O*-glucoside (63% of total anthocyanin content) [146]. However, in analog with callus culture, the grape cell suspensions usually consist of a mixture of uncolored cells and red colored anthocyanin producing cells [51, 142, 150, 151]. Moreover, the colorless cell fraction often has a high growth rate compared to the red fraction and this is the main factor that contributes to instabili-

ty of the supensions as regards anthocyanin production [150, 154]. With time, anthocyanin producing grape cell suspensions could significantly decrease their ability to produce anthocyanins and often the yields, obtained by the cultivation at equal conditions in the same cultivation vessels are unpredictable [142]. The other complication is due to the existence of strong correlation between cell differentiation, anthocyanin accumulation and cell growth [1]. Anthocyanins are produced only in cells that undergo some level of differentiation (growing as small aggregates), whereas the fast growing, undifferentiated fraction of cells (growing as single cells) does not produce the pigments [1, 155]. It was found that the fraction of aggregates larger than 0.6 mm accumulates high amounts of anthocyanins, whereas with the reduction of aggregate size to 0.2 mm the concentration of produced anthocyanins decreases with 50 % [155]. To control the ratios between non-pigmented and pigmented cells, as well as between aggregated and single cells, the period of sub-culturing and the size and age of used inoculums should be precisely adjusted [1, 150]. Optimization of the parameters for the inoculums has often been underestimated by researchers, working with plant cells. However, the establishment of right parameters for the inoculum has been found to have significant impact on growth and secondary metabolite production by different plant cell suspensions and hairy root cultures [3, 7, 156, 157]. The size of inoculum could significantly shorten the lag phase of grape cell suspension, as well as to affect the culture response to different changes in nutrient composition [158]. In our laboratory, we investigated the effect of different inoculum sizes on growth of anthocyanin producing *M. rotundifolia* var. "Noble" cell suspension cultures (Figure 9). The experiment was performed in submerged flasks by using 9, 15, 20 and 30 % (v/v) inoculum of 14 days old cell suspension. The maximum amount of accumulated fresh biomass (78.7±1.7 g FW/L) was achieved when 20 % of the inoculum was used. At these conditions, the amount of accumulated fresh biomass was 3.18-fold higher, compared to the experiment at which 9% of the inoculum was used, without observing any decrease in anthocyanin production (unpublished data).

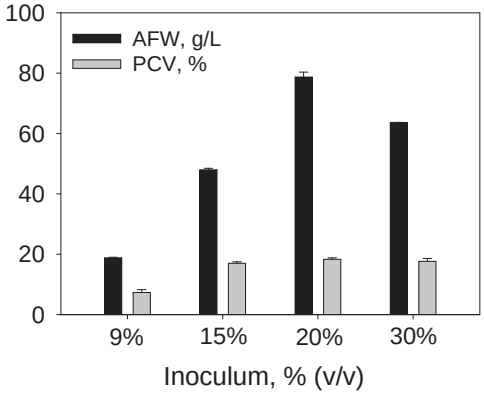

Figure 9. Effects of the inoculum sizes (14 days old culture) on accumulated fresh weight (AFW) and packed cell volume (PCV) by *M. rotundifolia* var. "Noble" cell suspension culture cultivated in shaking flasks (250 ml/50 mL) for 18 days. The presented values are means with standard deviations of two independent experiments repeated twice (n=4).

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However, the non-homogenous growth of grape cell suspensions could be a serious issue for large-scale cultivation of these in *vitro* cultures because of the complicated mass transfer in bioreactor systems. It has been observed that grape callus culture can undergo dramatic changes in their anthocyanin profiles, because of their high heterogeneity and somaclonal variability. To prevent those negative effects, a periodical implementation of deep phytochemical, genetic and metabolomic analyses are requires. This will retain acceptably high biosynthetic potential in selected lines during their maintenance.

5.2. Medium optimization

The optimal balance of nutrients in cultivation medium has been found to be an essential factor, determining the success on *in vitro* cultivation of plant cells and tissues. Plant cell suspensions are exceptionally sensitive to concentration of macronutrients, microelements, growth regulators, nitrogen and carbon sources. Even insignificant changes in composition of cultivation medium could promote the appearance of significant changes in cell morphology, growth and secondary metabolite profiles. For example, transferring of anthocyanin producing cell suspension of *M. rotundifolia* var. "Noble" from the original B5 medium into LS medium completely change the color pattern from red to yellow and the cell growth pattern from small colored aggregates to single colorless cells (Figure 10) (unpublished data).

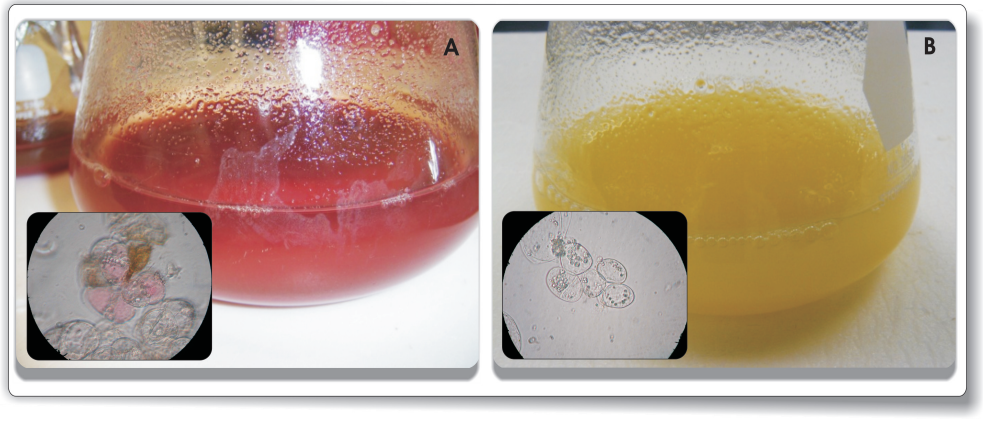

Figure 10. Cell suspension of *M. rotundifolia* var. "Noble" cultivated in shaking flasks on: A - B5 medium; B – LS medium.

As the grape cell suspensions exist as mixture of colored and colorless cells, the optimal cultivation medium should be developed by the way to provide a right balance between the growth rates of both cell populations [1, 142]. Finding the right nutrients balance is often a complicated task mainly because of the observation that the colorless population usually has better growth characteristics and the colored cells showed slow growth [1, 142, 150]. Dedaldechamp and Uhel [154] isolated a cell line from colorless cells of *V. vinifera* L. *cv.* Gamay Fréaux and demonstrated that under reduction of cell division by phosphate de-

ficiency, the anthocyanin production was reactivated in colorless cells [154]. Removing of phosphate ions from the medium leads to significant decrease of cell growth but remarkably increase anthocyanin production due to the increasing transcript levels of *UFGT* and *VvmybA1* genes [159]. Reduction of nitrate ions in the cultivation medium to a critical level was also found to enhance the anthocyanin production in cell suspension of *Vitis* hybrid Bailey Alicant A [160]. Increased osmotic pressure in medium, caused either by the increased sugar concentrations or by the addition of osmotic-active compounds as D-manitol, sorbitol, poliol or carboxymethyl cellulose has been found to play a critical role for anthocyanin production by different grape cell suspension cultures [155, 160-163]. Because of the significant differences in nutrient media compositions required to provide optimal cell growth and that to activate anthocyanin production, most of the authors used two-stage cultivation involving usage of "maintenance" medium, following by next transfer on "production" medium [1, 142, 146, 150, 160]. The maintenance medium provides rapid growth of the grape cell culture but the lines often lost their colors. When transferred on production medium, the growth was usually almost completely inhibited, but the anthocyanin accumulation was significantly enhanced. The production medium usually differs from the maintenance medium by the increased sugar concentration, decreased phosphate and nitrate concentrations, supplement of osmotic-active compounds, growth regulator compositions etc. [1, 142, 150, 146, 160]. However, it is obviously that the commercial realization of such two-stage based cultivation process is accompanied with numerous complications from technological point of view. In fact, this is one of the main reasons for the lack of industrial process for anthocyanins production by grape cell suspensions.

5.3. Effect of the pH

Since the pH is an important factor for anthocyanin stability and activities of enzyme systems in plant cells, its value in the cultivation medium is critically important for the regulation of both pigments and biomass yields. Recently, the effect of pH in culture medium was investigated on callus cultures of three grapevine varieties (Coarnă neagră, Fetească neagră and Cadarcă) [164]. The authors observed that the largest amount of accumulated anthocyanins (13.5 mg/g FW) were registered in callus culture of Fetească neagră, cultivated on the medium with the pH=4.5. When cultivated on medium with pH=9.0, the anthocyanin production by the same culture was significantly decreased (up to 3.2 mg/g FW) [164]. Suzuki and colleagues [162], investigated the growth and production of anthocyanin in grape cell suspension from *Vitis* hybrid Bailey Alicant A cultivated on media with different pH (4.5, 5.5, 7.0 and 7.5) [162]. The authors found that the best growth and pigment production were recorded when cultivation was done on medium with the pH=4.5. In contrary to that, when cultivated on medium with pH=7.0 the cell growth and the anthocyanin production were almost completely inhibited. Moreover, the percentage of pigmented cells were significantly decreased with the increase of pH (from 50% at pH=4.5 to 4% at pH=7.5) [162]. Because the pH value of culture medium during plant cell cultivation processes correlates with the metabolism of nitrogen sources, it is important to render an account of this factor during creation of experimental matrices for optimization procedures of nutrient medium composition (especially ammonia/nitrate ratio).

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5.4. Effect of temperature

Temperature has a strong effect on anthocyanin biosynthetic pathway, since some cold regulation genes are involved on it [165, 166]. Anthocyanin accumulation in berry skins of "Aki Queen" (*V. labrusca* x *V. vinifera*) after temperature treatment during ripening was significantly higher at 20 °C compared to that at 30 °C [167]. Similar effect was reported for berries of *V. vinifera* L. *cv.* Cabernet Sauvignon, where the anthocyanins concentration was reduced more than 50 % with the increase of cultivation temperature from 25 °C to 35 °C [168]. The authors suggest that the observed decrease in anthocyanin concentration under high temperature was as a result of both anthocyanin degradation and inhibition of mRNA transcription of the anthocyanin biosynthetic genes [168]. Recently, a qRT-PCR analysis have been carried out to compare the expression levels of MYB-related transcription factor genes in the berry skins of "Pione" (*V. vinifera* x *V. labruscana*) during temperature and light treatments [169]. The authors demonstrated that both low temperature and light irradiation were needed to induce the expression of *V1mybA1-3* (gene, encoding MYB-related transcription factors that regulate anthocyanin biosynthesis pathway genes), whereas the expression of *Myb4* (the repressor of UFGT) was up-regulated only by high temperature, independently of the light levels [169]. However, in optimization procedures for anthocyanin production by grape cell suspension cultures, the influence of temperature was underestimated. The new data, discovering the importance of those critical parameters in regulation of anthocyanin biosynthetic pathways outline the necessity for conduction of more experiments concerning optimization of temperature during cultivation of grape cell suspensions.

5.5. Light

Light is an important controlling agent in anthocyanin biosynthesis [166]. Light has been found to induce the expression of genes, responsible for activation of the promoters of the flavonoid pathway genes (*MybpA1*), but it has no effect on expression of repressor of UFGT (*Myb4*) in grape berries of vine 'Pione' (*V. vinifera* x *V. labruscana*) [169]. Recently it was demonstrated that UV-A light significantly stimulate the expression of structural genes, encoding the entry enzymes of the shikimate pathway, whereas only the UV-B and UV-C irradiation triggers the production of various anthocyanins in grape berries of *V. vinifera* L. *cv.* Cabernet Sauvignon [170]. Illumination of grape callus and cell suspension cultures is of great importance for expression of their anthocyanin producing potential. In our laboratory, we investigated the influence of light on anthocyanin production in callus culture of *M. rotundifolia* var. "Noble" (Figure 11). The red colored line was selected by 3 months repeated selection of dark red colored clusters, grown under illumination (16 h light : 8 dark). When the red colored line was transferred for cultivation in darkness, the culture completely lost its color for one sub-cultivation cycle (31 days). The obtained colorless culture was supported on darkness for 3 more sub-cultivation cycles and then transferred back under illumination (16 h light: 8 dark). Fourteen days after the light treatments began, the culture started to form red colored clusters and the total anthocyanin concentration reached the original levels, detected in initial red colored lines after 31 days of light exposure (unpublished data).

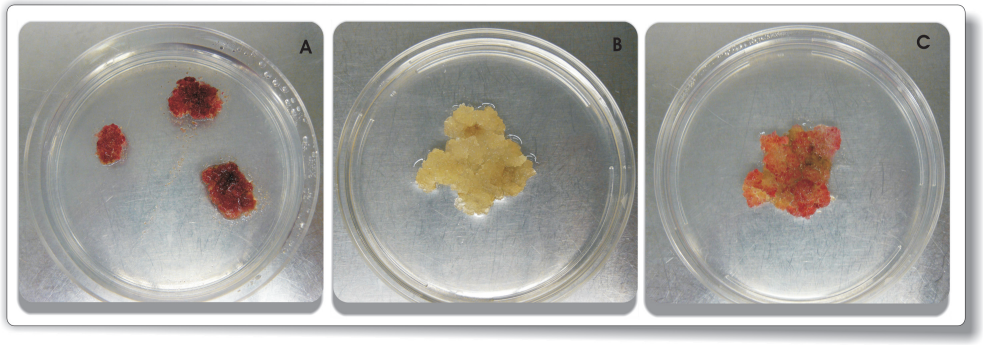

Figure 11. Anthocyanin accumulation in callus culture of *M. rotundifolia* var. "Noble": A - under illumination (16 h light : 8 dark); B – on darkness; C – roll-back under illumination (16 h light : 8 dark) of the culture, cultivated on darkness. The presented pictures are on the 14 days old cultures.

Lazar and colleagues investigated the effect of light on anthocyanin accumulation by callus cultures from six grapevine varieties (Burgund Mare, Cabernet Sauvignon, Merlot, Oporto, Negru Tinctorial and Pinot Noir) [171]. They found that the light has a stimulating effect on anthocyanin production in all calli studied, but the amount of accumulated pigments was in strong correlation with the genotype of variety used for callus initiation [171]. In grape cell suspension of *V. vinifera* L. *cv.* Gamay Fréaux, treatment with light leads to additional increase of peonidin-3-*O*-glucoside with 0.6 mg/g DW [172]. The same group reported that the continuous light irradiation (8000–8300 LUX) can contribute up to 4.8-fold increase in anthocyanin accumulation compared to no illuminated control [173]. Combined treatment with light and elicitor (jasmonic acid) additionally increased anthocyanins production (13.9-fold compared to non-treated control) [173]. It is obvious that the light requirements of grape cell suspension cultures can cause a serious complication during the scale up of cultivation process. Cormier and colleagues reported the isolation of red colored clusters growing on darkness, but the line has been very unstable and easily changed to variegated culture [142]. However the authors succeeded to obtain stable anthocyanin producing cell line in suspension culture but the production of pigments was relatively low (1.32 mg/g FW) [142]. Therefore, much effort is needed to initiate light-independent high anthocyanin producing cell lines, which will greatly facilitate the transfer of the process to large-scale bioreactors.

5.6. Effect of growth regulators

Availability of growth regulators (auxins and cytokinins) in cultivation medium are essential for ensuring the growth and to determine the levels of produced secondary metabolite by *in vitro* cultivated plant cells. In the case of anthocyanin biosynthesis in grape cell suspension, the composition of growth regulators in the medium can be of great importance for the production pigments [1]. Hirasuna and colleagues [160], tested the effects of different auxins (IAA, NAA, IBA and 2,4-D) and cytokinins (Kinetin and BAP) on anthocyanin biosynthesis by *Vitis* hybrid Bailey Alicant A cell suspension, cultivated on specially composed production

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medium [160]. The authors found that the synthetic auxin 2,4-D has stimulating effect on anthocyanin biosynthesis over a wide range of concentrations, whereas the addition of cytokinins even in low concentrations completely inhibit the pigments production. However, the effects of growth regulators are unpredictable and should be evaluated experimentally for every individual cell line. As an example, Krisa and colleagues [146], reported that for cell suspension of *V. vinifera* L. *cv.* Gamay Fréaux, the addition of NAA as auxin leads to better anthocyanin accumulation compared to when 2,4-D was used [146]. Moreover, their suspension culture requires the addition of cytokinin, Kinetin for ensuring the culture growth and production of pigments. The change of Kinetin with BAP results to inhibition of anthocyanin production [146]. Recently, Gagne and colleagues demonstrated that abscisic acid (ABA) promotes anthocyanin production in grape cell culture of *V. vinifera* L. *cv.* Cabernet Sauvignon (CS6) by the expression of some genes in the upstream part of the flavonoid biosynthesis pathway [174].

5.7. Effect of elicitors and precursors

Application of different (biotic or abiotic) elicitors has been proved to be an effective strategy for enhancement of the production of secondary metabolites related to the plant defense system [175]. Anthocyanin biosynthetic pathway as a part of phenylpropanoid metabolism of plant cells could be significantly manipulated by application of different elicitors or feeding with specific precursors. Treatment of cell suspension culture of *V. vinifera* L. *cv.* Gamay Fréaux with a combination of phenylalanine as a precursor and methyl jasmonate as an elicitor resulted in a 3.4-fold increase in anthocyanin yield compared to the control [151]. Cell suspension culture of *V. vinifera* L. *cv.* Gamay Fréaux was recently used as a model system for evaluating the effects of different chemical (streptomycin, activated charcoal, ethephon, indanoyl-isoleucine and N-linolenoyl-l-glutamine), biotic (insect saliva, chitosan, pectin, alginate, yeast extract and gum arabic) and physical (hydrostatic pressure and pulsed electric field) elicitors on anthocyanin biosynthesis [176-180]. It was found that the combined treatment of samples with pulsed electric field and ethephon, lead to a 2.5-fold increase in anthocyanin content, whereas the combination of hydrostatic pressure and ethephon does not alter anthocyanin production, but increases the other flavonoids [176, 177, 180]. The application of indanoyl-isoleucine enhanced the anthocyanin production with 2.6-fold, whereas the insect saliva stimulated the production of phenolic acids [178]. In contrast to insect saliva, the other investigated biotic elicitors (chitosan, pectin, and alginate) had a significant effect on anthocyanin production (resulted on 2.5-fold, 2.5-fold, and 2.6-fold increase compared to control, respectively) [179]. The most widely used elicitors are methyl jasmonate and jasmonic acids, which seems to have better beneficial effects on accumulation of phenolic acids and stilbenes than the accumulation of anthocyanins [181, 182]. However, jasmonic acid was found to increase preferably the level of peonidin-3-*O*-glucoside (from 0.3 to 1.7 mg/g DW) and the relative share of acylated anthocyanins (from 32% to 45%) in cell suspension of of *V. vinifera* L. *cv.* Gamay Fréaux, whereas the concentrations of the other major anthocyanins were insignificantly increased [172]. The effect of jasmonic acid was significantly increased when combined treatment with light irradiation was applied [172]. Magnesium treatment of cell suspension of *V. vinifera* L. *cv.* Gamay Red was found to increase the anthocyanin concentration

by inhibiting the degradation of pigments but not by promoting induction of biosynthetic-related genes [183].

6. Commercialization and applications of grape cell products

The increased demand of natural colorants and nutraceuticals determines the needs for development of alternative technologies for supply of such additives. The anthocyanins, produced by grape cell suspensions, represent a very attractive class of natural compounds, which could find application in food industry (as colorants), pharmacy (as nutraceuticals and therapeutic compounds) and in cosmetics (as UV protectors, antioxidant and anti cancer compounds). Biotechnological production of grape anthocyanins presents significant economical benefits. Cormier and colleagues calculated that the cost of 1 kg anthocyanins, produced by two-stage cultivation process of grape cell suspension in bioreactor with working volume of 155,000 L can cost almost the half of the price of such amount of anthocyanins, produced by the extraction of grape skins ($ 931 per 1 kg of anthocyanins from grape cell suspension, compared to $ 2,083 per 1 kg of anthocyanins, produced by grape skin extraction) [142]. However, the specific requirements of the available grape cell suspensions significantly complicate the scale up of the cultivation process, which is the serious restriction for realization of such biotechnological process.

6.1. Pharmaceutical applications

Anthocyanins have great potential for application in pharmaceutical products both as nutraceuticals and as therapeutic compounds. Frequent ingestion of anthocyanins could provide various health benefits including reduced risk of coronary heart diseases, anti-carcinogenic activity, antioxidant activity, reduced risk of stroke, anti-inflammatory effects etc. [13, 34, 184-186]. Biological activities of anthocyanin pigments have been already discussed in several excellent reviews [184, 187, 188]. Their pharmaceutical value has been additionally increased due to their high bioavailability. However, the administration and metabolism of anthocyanins *in vivo* have been investigated in details mostly in rats, whereas the detailed studies on humans still are scantly presented in scientific literature [60, 189]. For better understanding and investigation of anthocyanins absorption and *in vivo* metabolism in human and animal bodies, grape cell suspension culture of *V. vinifera* L. *cv.* Gamay Fréaux, was adapted to produce ^{13}C-labeled anthocyanins (delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside **7**, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside) [190]. Development of reliable sources of isotopically labelled anthocyanins could have remarkable impact on advancement in diagnostic of metabolomic assimilation studies of these compounds *in vivo*.

6.2. Food industry

The world market of natural food colorants expands with the annual growth rate of 4-6% [142]. In USA 4 of the 26 colorants approved by the food administration, that are exempt from certification, are based on anthocyanin pigments [34]. In European Union, all anthocyanin-

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containing colorants are classified as natural colorants under the classification E163 [191]. Currently most of the worldwide anthocyanins supply comes from processing of grape pomace, which is a waste product from winemaking. But in European Union other plant sources such as red cabbage, elderberry, black currant, purple carrot, sweet potato, and red radish are also allowed [192]. Anthocyanins, produced by grape cell suspensions can be a promising alternative supply of natural colorants. It has already been demonstrated that the produced pigments by the grape cell suspensions undergo significant structural modifications. Grape cell suspensions accumulates higher levels of metabolically more evolved structures (methylated and acylated anthocyanins). Acylated anthocyanins are suitable for application in food products, mainly because of the improved color stability compared to non-acylated structures [72]. Moreover, the grape cell suspensions can also produce elevated levels of beneficial phenolic compounds such as flavonoids, stilbenes, phenolics, etc., which are capable of increasing the added value of the final additive. The overall metabolite profile of grape cells in combination with the lack of microbial and toxic contaminations will give the potential for development of new types of food additives if the entire cell suspension biomass are utilized.

6.3. Cosmetic industry

The commercial interest of cosmetic companies to apply plant additives, derived by biotechnological cultivation of plant cells to their products has increased remarkably in the last few years [193]. The addition of plant cell derived extracts in cosmetic products has been considered as a powerful approach used to increase their health benefits. Several plant extracts have been added to various cosmetic products as moisturizers, antioxidants, whitening agents, colorants, sunscreens, preservatives etc. [193]. With the advancement of plant cell biotechnology, more and more cosmetic companies have been attracted for application of additives, based on plant cell suspensions. Recently the application of so-called plant "steam" cells attracts industry's attention [193]. In the last few years, the French company "Sederma" launched the product "Resistem™" based on application of *in vitro* cultivated plant cells (www.sederma.fr). The other company, "Mibelle Biochemistry", situated in Switzerland, developed a "PhytoCellTec" product, based on grape cell suspension of *V. vinifera* L. *cv.* Gamay Fréaux, which was processed by high-pressure homogenizer to produce liposomes for application in cream products (www.mibellebiochemistry.com). According to the company, the grape cell derived liposomes contained higher amounts of anthocyanins and when applied on skins serve as strong UV protectors and fight photoaging. The presented examples clearly demonstrate the commercial interest to application of grape cell suspension derived products. However, it is a matter of time for the scientists to develop the biotechnological approach of producing anthocyanins by grape cell suspensions from the frame of experimental scale to commercially applicable products.

7. Conclusion and future prospects

The approaches described in this chapter can be effective in improving novel anthocyanin-derived metabolites in grape cell suspensions. Continuous study and exploitation of the knowledge of grape cell lines and their control mechanisms will open up new possibilities for

metabolic engineering of the anthocyanin biosynthesis pathway. In parallel, the recent achievements in bioengineering with plant cell suspensions and the improvements of the existed bioreactor designs discovers new prospectives for commercial realization of anthocyanin producing technology based on cultivation of grape cells. This is a research area that is growing and gaining interest in the analysis of plant-based health-related compounds. Therefore, the full impact of metabolomics on muscadine research is yet to be experienced. But this chapter serves as a starting point for scientists who are interested in cell cultures from muscadine grapes.

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