Tobacco Cell Suspension Cultures: An Eco-sustainable Approach for Obtaining Stable Anthocyanins

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Short Communication

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INTRODUCTION

Anthocyanins are a class of water-soluble flavonoids widely distributed in nature and responsible for the pigmentation of various plant organs. In recent years, interest in these secondary metabolites has significantly increased, particularly due to their potential applications for nutraceutical, cosmeceutical, and medical purposes. Although most publications report the extraction of these bioactive compounds from whole plants, plant cell cultures represent an eco-sustainable alternative for obtaining these flavonoids with extremely high yields through scalable and standardized processes. This article aims to focus on the advantages of anthocyanins production using engineered *Nicotiana tabacum* cells, which enable the extraction of a high-value product in terms of stability and potential applications.

Abbreviations: C3G: Cyanidin-3-O-Glucoside; PDSMs: Plant Derived Secondary Metabolites; CGA: Chlorogenic Acid; C3R: Cyanidin-3-Rutinoside; ACNs: Anthocyanins

DESCRIPTION

Advantages of anthocyanins production in plant cell suspension cultures compared to extraction from whole plants

Plant cell suspension cultures represent a simple and cost-effective system for obtaining plant-derived secondary metabolites (PDSMs). This technology offers numerous benefits compared to other methods, such as extraction from whole plants, including higher yields, excellent scalability, and the ability to prevent the overexploitation of plant species [1].

This production system represents the basis of the process through which ABResearch obtained a plant extract from an engineered tobacco cell line (*Nicotiana tabacum* M1B3) that overexpressed both peach genes PbMYB10.1 and PpbHLH3, transcription factors involved in the anthocyanins (ACNs) biosynthetic pathway. The extract, derived from *Nicotiana tabacum* transgenic cells, was concentrated, purified and freeze-dried. And it was named ANT-CA because of its content of 10% ACNs and 30% chlorogenic acid (CGA), while the freeze-dried cells had an ACNs content of 25 mg/g [2]. Currently, this production process allows the extraction of, on average, 100 mg of ACNs and 300 mg of CGA per gram of freeze-dried extract.

The production of anthocyanins in in-vitro plant cells or tissue cultures derived from various plant sources has been reported in several publications, as summarized by Belwal et al. The most extensively studied species for this purpose include *Vitis vinifera*, *Rosa hybrida*, and *Daucus carota* [3]. Additionally, other researchers have already evaluated the use of engineered tobacco cell suspensions to produce these flavonoids. Appelhagen et al., for instance, reported a yield of 90 mg L-1 of anthocyanins titrated as cyanidin-3-O-glucoside equivalents (C3G) by culturing genetically modified *Nicotiana tabacum* cells in 2 L fermenters [1]. These productivity data are comparable to those obtained by ABResearch in 25 L pilot-scale bioreactors (125 mg C3G equivalents L-1 culture medium).

Most publications regarding ACNs extraction from plant sources, however, utilize whole plants. Pedro et al., for example, evaluated the recovery of these polyphenols from black rice, obtaining 116.58 mg 100 g^{-1} of anthocyanins [4]. Similarly, the extraction of anthocyanins from purple corn flour, by Ursu et al., yielded 14.04 ± 0.02 mg cyanidin-3-O-glucoside equivalents (C3G) /g dry weight (DW) [5]. Finally, it is possible to extract 108.23 mg/100 g DW of these biomolecules from blueberries, as demonstrated by Zhang et al. [6].

However, the use of such plant sources for anthocyanins production entails an inevitable consumption of land and water resources. Moreover, conventional agricultural practices, such as flooding and irrigation systems, removal of unwanted vegetation, and the use of herbicides, pesticides, and fertilizers, have led to a significant decline in biodiversity over the past 50 years [7]. The production of PDSMs from plant cell cultures, compared to the conventional extraction of these compounds from whole plants, is independent of seasonal variations, has a reduced carbon footprint, and does not require herbicides or pesticides [8]. The use of this technology presents itself as an eco-sustainable solution for large scale ACNs production. In fact, plant cell cultures are easily industrial-level scalable, enabling anthocyanins production comparable to that obtained from conventional agricultural sources. From this perspective, ABResearch has the capability to use 1000 L fermenters, which currently allow the production of 125 g of anthocyanins and 375 g of chlorogenic acid per operational unit, within an area of approximately 1 m^2 [2]. The average yields per square meter for corn and rice are 1400 g/m^2 and 1000 g/m^2, respectively [9,10]. Assuming ACNs production in these conventional crops, the yields per square meter would be 19.66 g for corn and 1.17 g for rice, based on the anthocyanin extraction yields from black rice and purple corn reported earlier. Considering that up to 25 production batches per year could be generated in the same space occupied by a fermenter, it becomes evident that this technology leads to extremely high yields (3125 g of anthocyanins and 9375 g of chlorogenic), especially compared to traditional crops, many of which give only a single annual harvest.

Solutions aimed at obtaining natural pigments, such as anthocyanins, in an eco-sustainable way, are particularly important given the rapidly growing market segment for natural dyes and pigments, which is estimated to reach approximately US$ 24.4 billion in 2024 and US$ 41.4 billion by the end of 2034 [11].

Cyanidin-3-Rutinoside Properties

Anthocyanins are defined as the glycosylated forms (aglycones) of anthocyanidins. The glycosylation of anthocyanins can occur at various hydroxyl moieties, with 3-OH being the predominant glycosylation site in nature, forming 3-O-β-glucosides [12]. The six most commonly occurring anthocyanidins in higher plants are pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt), and delphinidin (Dp). The glycosylated forms of the three non-methylated anthocyanidins (Cy, Dp, and Pg) are the most abundant in nature [13]. The extraction of anthocyanins from various plant sources reveals that their content varies significantly depending on the variety and predominant species, as analysed in multiple publications [14,15].

In contrast, *Nicotiana tabacum* M1B3 cells, from which ANT-CA was obtained, predominantly produce cyanidin-3-rutinoside (C3R), which constitutes 72.5% of the total anthocyanins content identified in the freeze-dried extract [2]. C3R is more stable compared to other anthocyanins. Sui et al., for example, reported higher resistance of cyanidin-3-rutinoside to degradation compared to cyanidin-3-glucoside, in response to variations in temperature and pH [16]. Additionally, C3R has various medical applications. Feng et al. has proposed this flavonoid as a potential therapy for leukemia, peaks its selective action on cancer cells by inducing a paradoxical pro-oxidative effect [17]. Cyanidin-3-rutinoside could also have a therapeutic role in diabetes treatment by counteracting hyperglycemia-induced β cell damage through the reduction of glucotoxicity induced apoptosis in INS-1 pancreatic β cells [18]. This makes this compound of particular interest for future studies and applications.

Synergistic Effect of Co-Pigmentation with Chlorogenic Acid

At low pH values, anthocyanins exist as stable flavylium cations, while at more basic pH, they can undergo various degradative pathways, leading to colour loss [12]. At pH 5-6, the flavylium cation is easily hydrated and converted into the colourless hemiacetal [19].

One of the most widely used techniques to enhance ACNs stability is co-pigmentation. This technique involves the formation of non-covalent complexes between anthocyanins and co-pigments such as polymers, carbohydrates, and phenolic compounds [20]. Specifically, co-pigmentation leads to the formation of a sandwich configuration between anthocyanin and co-pigment, held together by hydrophobic interactions ($\pi - \pi$ stacking). This complex can protect the flavylium cation of ACNs complexes from degradation, preventing hydration and intensifying the red colour of the solution [19]. Chlorogenic acid is one of the most commonly used phenolic compounds in co-pigmentation techniques for anthocyanins stabilization. For example, Azman et al. feature that chlorogenic and ferulic acids are among the best co-pigments compared to other phenolic compounds, leading to an increase in anthocyanin half-life at pH 3.0 and pH 6.0 of buffer solutions, respectively [21]. Moreover, the study by Gras et al. (2018), report that the addition of CGA increased the absorbance of non-acetylated anthocyanins extracted from black carrot by 97.9% and 122.9%, at pH 3.6 and 4.6 respectively. This effect could be attributed to the formation of complexes between ACNs and chlorogenic acid [22].

In addition to anthocyanins, the ANT-CA extract from *Nicotiana tabacum* M1B3 cells contains a high concentration of polyphenols, in particularly chlorogenic acid (30% of the freeze-dried extract) [2]. The significant presence of CGA in the lyophilized extract may thus justify the high stability of the anthocyanins present. Furthermore, chlorogenic acid generates several therapeutic effects, reducing many chronic inflammatory and age-related disorders through anti-inflammatory, antioxidant, and metabolic homeostasis modulating actions [23]. Its pharmacological properties

include hepatoprotective, antimicrobial, immunomodulatory, antioxidant, and antitumor activities. Several studies emphasize CGA antitumor activity through cell cycle inhibition, apoptosis activation, and tumor cells suppression [24]. Therefore, it is plausible to assume that the presence of chlorogenic acid in the ANT-CA extract may generate a synergistic effect with anthocyanins, both in terms of stability and potential therapeutic actions.

CONCLUSION

Based on plant cell suspension technology, ABResearch has obtained an engineered tobacco cells extract containing 10% anthocyanins and 30% chlorogenic acid. This yield is extremely high compared to ACNs extraction from several plants, either as a whole or from their parts. Most of the anthocyanins present in ANT-CA are represented by cyanidin-3-rutinoside (72.5%), which has a high therapeutic potential. This flavonoid is reported to have higher stability than other ACNs, a property significantly enhanced by co-pigmentation with chlorogenic acid, which is abundantly present in the extract. CGA also has therapeutic properties, with possible medical applications. The use of *Nicotiana tabacum* M1B3 suspension cells enables the production of these PDSMs, in a sustainable and eco-friendly way, with high yields and relatively low costs, addressing the growing demand for natural dyes and pigments.

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