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REVIEW

Tocopherol production in plant cell cultures

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Tocopherols, collectively known as vitamin E, are lipophilic antioxidants, essential dietary components for mammals and exclusively synthesized by photosynthetic organisms. Of the four forms (α, β, γ and δ), α-tocopherol is the major vitamin E form present in green plant tissues, and has the highest vitamin E activity. Synthetic α-tocopherol, being a racemic mixture of eight different stereoisomers, always results less effective than the natural form (*R,R,R)* α-tocopherol. This raises interest in obtaining this molecule from natural sources, such as plant cell cultures. Plant cell and tissue cultures are able to produce and accumulate valuable metabolites that can be used as food additives, nutraceuticals and pharmaceuticals. Sunflower cell cultures, growing under heterotrophic conditions, were exploited to establish a suitable *in vitro* production system of natural α-tocopherol. Optimization of culture conditions, precursor feeding and elicitor application were used to improve the tocopherol yields of these cultures. Furthermore, these cell cultures were useful to investigate the relationship between α-tocopherol biosynthesis and photomixotrophic culture conditions, revealing the possibility to enhance tocopherol production by favouring sunflower cell photosynthetic properties. The modulation of α-tocopherol levels in plant cell cultures can provide useful hints for a regulatory impact on tocopherol metabolism.

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1 Introduction

Tocopherols and tocotrienols, commonly known as vitamin E, are essential nutrients for humans and animals [1]. They consist of a hydrophilic chromanol head and a hydrophobic isoprenoid side chain. Tocopherols and tocotrienols differ only in the degree of saturation of their hydrophobic tail. Each group is composed of four members differing in the number and position of methylation on the aromatic ring, named as α-, β, γ, and δ-form [2]. Functionally, tocopherols and tocotrienols are lipid antioxidants with the ability to directly quench activated oxygen species or indirectly terminate lipid peroxidation chain reaction, by removing polyunsaturated fatty acid radical species [3, 4]. Other functions have been reported in mammals and, more recently, also in plants, including the participation in intracellular signalling [5–7].

Tocopherols and tocotrienols can be synthesized only by photosynthetic organisms, including plants and di-oxygenic photosynthetic bacteria. Tocotrienols are mainly present in certain seeds and cereals, while tocopherols are ubiquitously present, even if at different concentrations, in oil seeds, leaves and other green parts of higher plants [4].

In higher plants the biosynthesis of tocopherols takes place in plastids and in the last decade all the genes involved in this pathway have been identified in both plants and cyanobacteria, making it possible to elucidate this important pathway. Figure 1 shows the tocopherol biosynthetic pathway starting from its first true step, the condensation of the precursors homogentisic acid and phytyl pyrophosphate, the former providing the head group, the latter the hydrophobic tail [8].

Of the four forms, α-tocopherol has the highest vitamin E activity, possibly due to a preferential absorption and distribution of this molecule in the human body. Naturally

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synthesized α-tocopherol is a single (*R,R,R*) stereoisomer while chemically synthesized α-tocopherol, the most common form in vitamin E supplements, is a racemic mixture of eight stereoisomers, which is less effective than the natural (*R,R,R*) α-tocopherol [2, 4]. This raises interest in obtaining such a molecule from natural sources. In plants, α-tocopherol predominates in green tissues, γ-tocopherol being the main form in seeds. However, wheat germ oil, safflower oil and sunflower oil are especially rich in *(R,R,R)* α-tocopherol [9].

Tocopherol production from whole plant tissues is strongly influenced by seasonal variations limiting the time of plant growth and seed maturation. Another source of variability is given by environmental conditions (water availability, mineral nutrition, *etc.*), which strongly affect plant yield. An increase of vitamin E content has been obtained in engineered plants. The strategies used were aimed at improving tocopherol composition by converting most of the produced forms to the most active α-tocopherol or enhancing the concentration of the tocopherol and tocotrienol pools by increasing the flux through their biosynthetic pathway. According to the engineered plant species, the gene source and the engineering approach, the range of successful results was very variable, since an increase of vitamin E content from a 2% to a 20-fold has been reported (for a review, see [10]). Nevertheless, engineered plants do not avoid the criticism of seasonability and environmental constraints.

On the other hand, plant cell and tissue cultures represent a potential source of valuable metabolites, which can be used as food additives, nutraceuticals and pharmaceuticals, on a continuous year-round basis and using small spaces. They can also be useful tools for investigating plant metabolic pathways. Moreover, the production of phytochemicals by cell cultures can ensure a uniform quality, highly specialized, natural compounds that cannot be produced in equal quality or specificity by chemical synthesis. Such a production is also independent of environmental conditions and quality fluctuations, which can affect field-grown plants [11, 12]. In recent decades, the interest in chemopreventive plant natural products, such as antioxidants, has grown rapidly. This is due to the increasing evidence pointing to the involvement of oxidative stress in the pathogenesis of various human degenerative diseases and conditions [13].

The present work reports on the exploitation of plant *in vitro* cell cultures for investigating the production of tocopherols.

2 Establishment of *in vitro* cell cultures

Plant tissues vary enormously in their tocopherol content and composition (Table 1) [8, 14–20]. For establishing cell cultures with the aim of vitamin E production, the tocopherol composition of starting tissues is to be considered, together with the tissue suitability for *in vitro* manipulation. Sunflower (*Helianthus annuus* L.) cell cultures have been the most investigated *in vitro* system for tocopherol production. Callus and cell suspension cultures were induced from plantlets where α-tocopherol was always more than 91% of the total tocopherols. It is interesting to note that such a percentage was maintained in cell cultures and the average α-tocopherol content was similar to that measured in the starting tissue (Table 2) [21, 22]. Also callus cultures induced from safflower (*Carthamus tinctorius*) flower buds mainly accumulated α-tocopherol [23]. Recently, the establishment of seedling-derived callus cultures able to produce only α-tocopherol has been reported from two pseudocereal species, *Amaranthus caudatus* and *Chenopodium quinoa*, although the production levels were much lower than in plants [24].

It is known that several chemical and physical factors can affect the biosynthetic pathways of plant metabolites [12]. Optimal culture conditions were established for heterotrophic callus and suspension cultures of sunflower, in terms of cell growth rate and α-tocopherol production. The most efficient medium was found to be MS basal medium containing naphthaleneacetic acid and 6-benzylaminopurine with the addition of casaminoacids and myo-inositol. Furthermore, a 78% increase of α-tocopherol in cell suspensions in the stationary phase was obtained by selecting cells for longer subculture intervals, probably due to the response to a stress condition (Table 2) [22]. The ability of casaminoacids and myo-inositol to enhance tocopherol production had already been observed in safflower cell cultures [23].

3 Cell line variability

Plant cell cultures are often characterized by a certain degree of variability, which can be useful to identify highly productive cell lines. The cell line selection can be an important step in exploiting plant cell cultures as ''cell factories'' for valuable compounds [25]. Two cell lines of sunflower with differing capability to synthesize α-tocopherol were identified. In spite of the differing content of α-tocopherol (almost threefold higher in the high synthesizing cell line, HT, than in the low synthesizing one, LT), these cell lines had comparable growth curves. It is interesting to note that HT cells also produced higher levels of another antioxidant vitamin, ascorbic acid and the tripeptide glutathione. On the other hand, LT cells balanced their lower levels of antioxidant molecules by increasing the activities of some antioxidant enzymes, such as ascorbate peroxidase and catalase [21]. The availability of plant cell lines with enhanced biosynthesis of antioxidant molecules is a good tool for studying the regulation of their synthesis and the homeostatic responses, occurring as a consequence of the alteration of their content in the cells.

Table 1. Tocopherol content of fruits, vegetables, legumes, cereals and oil seeds

Food	Total tocopherols (μg/g FW)	α-Tocopherol (%)	Reference
Fruits			
Apples	4.3	75	[14]
Avocado	13.0	92	[14]
Bananas[a)]	84.0	2	[15]
Grapes	7.0	86	[15]
Oranges	4.0	100	[15]
Peaches	11.0	91	[15]
Pears	1.1	91	[15]
Plums	8.0	87	[16]
Raisins	4.3	93	[14]
Strawberries	8.0	75	[15]
Vegetables			
Beets	1.0	100	[15]
Broccoli	7.0	71	[17]
Cabbage	0.2	100	[14]
Carrots	4.2	95	[14]
Potatoes	0.7	90	[8]
Lettuce	9.0	67	[15]
Peppers	6.2	97	[14]
Spinach	30.0	63	[8]
Legumes			
Beans[b)]	4.8	27	[15]
Peas[c)]	16.7	1.7	[15]
Cereals			
Wheat kernel[d)]	50.0	20	[8]
Rice (white grains)[e)]	17.0	18	[8]
Corn kernel[f)]	60.0	10	[8]
Oil Seeds			
Rapeseed[g)]	314	36	[18]
Soybean[h)]	323	18	[19]
Sunflower	667	90	[20]

a) 4% β-tocopherol and 94% δ-tocopherol.
b) 67% γ-tocopherol and 6% δ-tocopherol.
c) 95.8% γ-tocopherol and 2.5% δ-tocopherol.
d) 56% β-tocotrienol, 15% β-tocopherol and 10% α-tocotrienol.
e) 30% α-tocotrienol, 30% γ-tocotrienol and 10% γ-tocopherol.
f) 75% γ-tocopherol, 15% α-tocotrienol and 15% γ-tocotrienol.
g) 63% γ-tocopherol.
h) 62% γ-tocopherol and 20% δ-tocopherol.

4 Enhancement of tocopherol production

Special treatments, including precursor feeding and elicitor application, can be used to increase metabolite production by plant cell cultures. When the biosynthetic precursor of the polar head, homogentisic acid, was administered to sunflower suspension cultures, a clear stimulatory effect (30% increase) on the production of α-tocopherol was observed, suggesting that this compound can be useful for improving the yield of sunflower cell cultures. On the other hand, the precursor of the hydrophobic tail, phytol, could not increase α-tocopherol production on its own [22].

A considerable enhancement of α-tocopherol production was achieved, both in sunflower and *Arabidopsis thaliana* cell cultures, by the exogenous application of 5 μM jasmonic acid [26] or by hypoxic conditions (Caretto, unpublished) (Fig. 2). Jasmonic acid is considered a key compound of the signal transduction pathway involved in several plant stress responses. The exogenous application of jasmonic acid is known to elicit the production of a wide range of compounds by inducing the expression of plant genes for various biosynthetic pathways [27, 28]. Probably, the observed enhancement of tocopherol production in sunflower and *Arabidopsis* cell cultures could be due to the ability of the exogenously added jasmonic acid to upregulate

Table 2. α-Tocopherol levels of different sunflower tissues

Source	α-Tocopherol (μg/g FW)
Hypocotyl	11.4 ± 0.8
Stem	7.3 ± 0.6
Leaf	18.1 ± 1.3
Hypocotyl-derived cultures	
Initial calli	12.1 ± 1.1
Standardized calli	19.8 ± 2.9
Initial suspensions	13.4 ± 1.4
Standardized suspensions	24.0 ± 1.2

Values are the means of three independent experiments ± standard deviation.

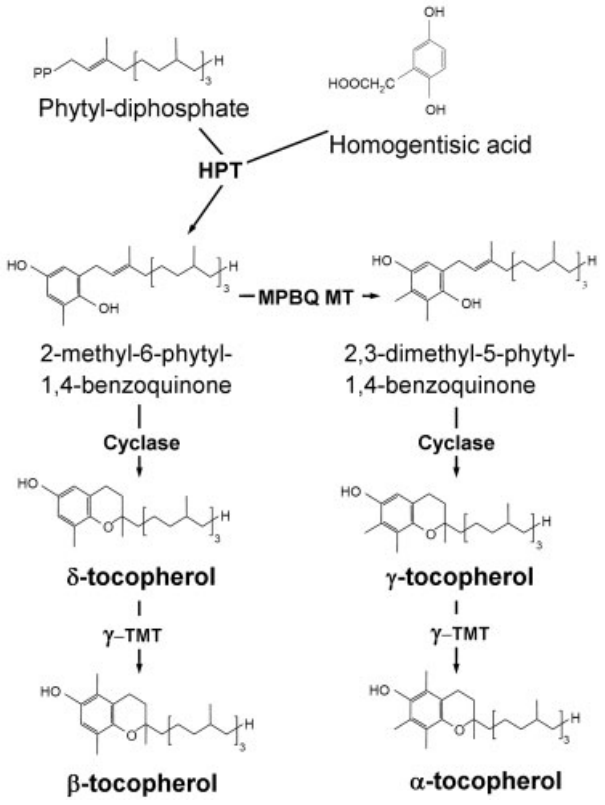

Figure 1. Tocopherol biosynthesis in plants. HPT: homogentisate phytyltransferase; MPBQ MT: 2-methyl-6-phytyl-1,4-benzoquinone methyltransferase; cyclase: tocopherol cyclase; γ-TMT: γ-tocopherol methyltransferase.

the biosynthetic pathway through the induction of gene expression. Gene expression analysis revealed that the levels of two genes of the tocopherol biosynthetic pathway, *p*-hydroxyphenyl pyruvate dioxygenase and homogentisate phytyltransferase, were indeed enhanced, compared with the control actin gene, after the jasmonic acid treatment of Arabidopsis cell cultures (Caretto, unpublished).

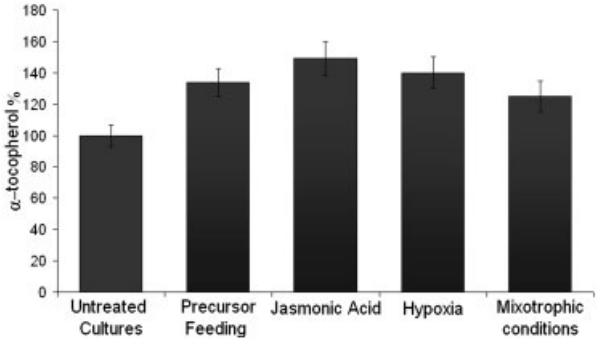

Figure 2. Enhancement of α-tocopherol production by various treatments in sunflower cell suspension cultures. Tocopherol levels are expressed as percentages of the control values ± standard deviation.

5 Tocopherol biosynthesis and photomixotrophic culture conditions

Among the various plant *in vitro* systems, photoautotrophic and photomixotrophic cell cultures can be suitable tools for investigating those physiological and molecular events that require intact chloroplasts [29]. In higher plants the biosynthesis of α-tocopherol is localized in plastids and more specifically in chloroplasts of photosynthetic tissues [30] and it is known that α-tocopherol is most abundant in plant photosynthetic tissues [3]. To investigate the relationship between α-tocopherol biosynthesis and photomixotrophic culture conditions, a new photomixotrophic sunflower cell line, HS3, was established by selecting cells able to grow in the presence of a tenfold reduced sucrose concentration in the culture medium. The photosynthetic properties of this cell line were compared with the starting cultures, revealing an increase in chlorophyll content, chloroplast number and level of the photosynthesis related enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Furthermore, an enhanced expression of the gene encoding for the tocopherol biosynthetic enzyme geranylgeranylpyrophosphate synthase was observed in HS3. This cell line also showed a significantly higher tocopherol level than the starting sunflower cells (Fig. 2), revealing the possibility to enhance tocopherol production by favouring the photosynthetic potential of sunflower cells [31].

6 Concluding remarks and future perspectives

Plant cell cultures, optimized for the *in vitro* production of α-tocopherol, can be considered promising "bio-factories" of natural vitamin E. Various strategies, such as media manipulation, precursor feeding and elicitor application are useful to improve the tocopherol yields of these cultures. Moreover, the availability of plant cell lines with enhanced tocopherol biosynthesis can be an important step in view of scaling up the

in vitro production system, by means of bioreactors. However, in order to assess plant cell cultures as a competitive and valuable alternative when compared with other natural vitamin E sources, more studies are needed for the industrial application of these achievements. In order to further improve vitamin E production, engineered plants with enhanced vitamin E levels could also be exploited as starting sources for establishing new highly productive cultured cell lines.

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