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PRODUCTION OF TOCOPHEROLS BY CELL CULTURE OF SAFFLOWER*

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Key Word Index—*Carthamus tinctorius*; Compositae; safflower; cell culture; tocopherol production; vitamin E; biotransformation; phytol; homogentisic acid.

Abstract—A new method for the production of tocopherols by safflower (*Carthamus tinctorius*) cell culture has been developed. The main tocopherol produced is α-tocopherol which has the strongest vitamin E activity among tocopherol analogues. In a time course experiment tocopherol production showed a secondary metabolic pattern rather than a primary one. Better cell lines in terms of both growth rate and amounts of tocopherols produced were obtained by selection using various growth regulators and media additives. In addition, tocopherol production was effectively stimulated by administration of biosynthetic precursors. In particular, phytol increased the total tocopherol content by some 18-fold, i.e. 63.6 mg per 100 g dry weight, and the α-tocopherol content by some 11-fold i.e. 28.8 mg per 100 g dry weight.

INTRODUCTION

Alpha-, β-, γ- and δ-tocopherols occur naturally in many kinds of vegetable oils (Scheme 1). Although the four analogues have different detailed actions and activities, all of them commonly have the physiological action of vitamin E [1] and an antioxidant action [2–4]. Tocopherols are used as ingredients of pharmaceutical preparations and as antioxidants in food [5, 6]. It has been found that tocopherols may have a bodily-condition deterioration-preventing or geriatric disease-preventing action [7–9], and accordingly, the demand for tocopherols obtained from vegetables is rapidly increasing.

Tocopherols are obtained by chemical synthesis or by extraction from vegetable sources. However, the compounds obtained by chemical synthesis are racemic (*dl*)-, whereas those from natural sources are the *d*-isomers [10]. The physiological activity of (*d*)-α-tocopherol, the most active tocopherol, is 1.36 times greater than that of racemic (*dl*)-α-tocopherol. Therefore, tocopherols from a natural source are to be preferred. As described above, tocopherols occur in vegetable oils such as soy bean oil, cotton seed oil and the like [11]. However, these vegetable oils generally have a low content of tocopherols. Therefore, the production at low cost of tocopherols from a vegetable source wherein the physiologically most effective α-tocopherol is present in large amounts is strongly desired.

The present paper provides a new process for the production of tocopherols by cell culture of *Carthamus tinctorius* L. (safflower) a plant which contains mainly α-tocopherol.

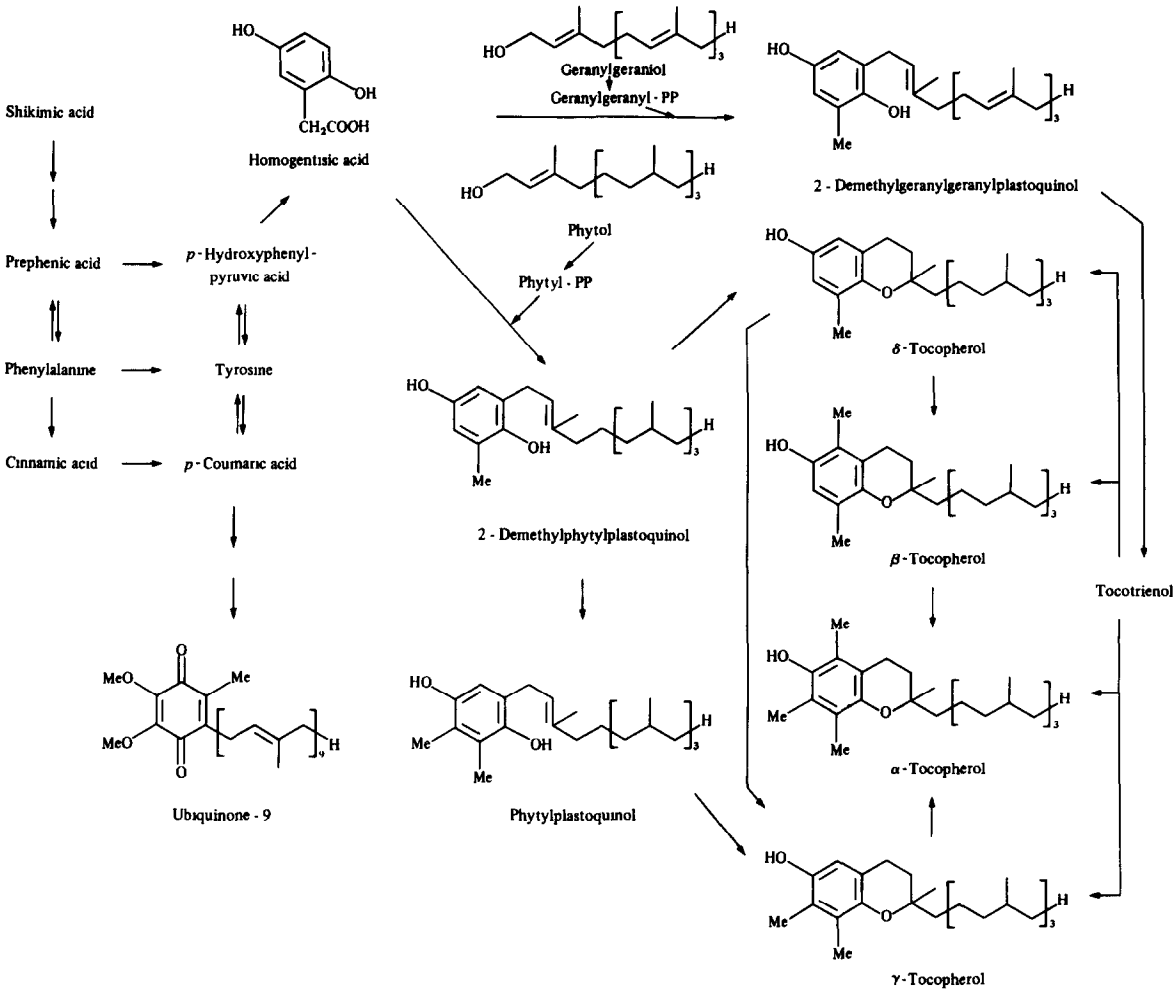
RESULTS

Growth of, and tocopherol production in, safflower cell cultures

Safflower callus which has been subcultured at 3-week intervals on Murashige and Skoog's medium supplemented with 1 ppm 2,4-D and 0.1 ppm K (abbreviated as DK medium) for nine years was cultured on a variety of media for 3–5 generations, and then the growth ratio and the tocopherol content of each callus were measured (Table 1). All the calli contained α-tocopherol as the main tocopherol and β-tocopherol, but no γ- or δ-tocopherol. The requirement of an auxin and/or cytokinin for growth and tocopherol production was not essential, because in hormone-free medium both the growth and tocopherol content were higher than those in the original callus (D 1 K 0.1 MS medium). On the other hand, calli cultured on Revised tobacco (RT) medium containing 0.1% casamino acid showed higher values for both the growth ratio and tocopherol content in comparison with those cultured on Murashige and Skoog's (MS) basal medium. In particular, the yellow callus cultured on RT medium supplemented with 2 ppm IBA (indole-3-butyric acid), 0.1 ppm kinetin and 0.1% CA (casamino acid) (B2KC medium) gave the highest growth ratio, (*ca* × 2) and α-tocopherol content, (*ca* × 5), when compared with the parent callus (D 1 K 0.1 MS medium).

To investigate the effective components in the B2KC medium, cell suspension cultures were set up for various liquid media (Fig. 1). The growth ratio and the tocopherol content were found to be almost the same in both MS and RT basal medium. Since the RT medium contained 55 times more inositol (0.55%) than the MS medium, the effects of inositol on growth and tocopherol production were investigated, singly or in combination with casamino acid. The results showed that inositol had least effect when added alone, but in the presence of casamino acid it was the most effective for both growth and tocopherol production.

* Part 49 in the series 'Studies on Plant Tissue Cultures'. For Part 48 see Furuya, T., Orihara, Y. and Hayashi, C. (1986) *Phytochemistry* **26**, 715.



Scheme 1.

The effect of casamino acid, from 0.1% to 1.0%, on growth and tocopherol production in safflower cell suspension cultures was investigated in detail. All the cells grew vigorously after transfer to a medium containing casamino acid, except at the 1% level which was inhibitory to both growth and tocopherol production. However, the addition of 0.1–0.5% casamino acid had almost the same effects on both growth, 8.0–9.8 times in ratio, and tocopherol content, 11.4–13.4 mg per 100 g dry weight. Therefore, using cells subcultured for several generations in suspension on the B2KC medium containing 0.1% casamino acid, the relationship between growth, as measured by dry weight increase, and tocopherol production was investigated next (Fig. 2). Cells (4 g) were inoculated into 250 ml medium contained in a litre flask. The cells were harvested every five days and their growth and tocopherol content determined. Maximal growth was achieved 20 days after inoculation, after which growth rapidly decreased. The tocopherol content of the cells remained almost constant at about 10 mg per 100 g dry weight in the lag and exponential phases of growth and then rose rapidly to 28.1 mg per 100 g dry weight at 25 days on day 5 after maximal growth. Alpha- and β-tocopherol were both produced throughout the culture period, the former accounting for some 73.0–88.6% of the total. No tocopherol was found in the medium throughout the culture period.

Increase of tocopherol content by administration of precursors

Callus (10–20 g) from three-week-old cultures was inoculated into 250 ml liquid medium (in a 1 l Erlenmeyer flask) and incubated at 26° for 1 week. Two-ml aliquots of the precursors dissolved in 20% Tween 80 solution at 12.5 mg per ml were administered to the cell suspensions under sterile conditions. After culturing for two weeks, the cells and media were separately extracted with *n*-hexane, and the amount of each tocopherol was determined by HPLC (Table 2). The precursors of the aromatic ring, apart from homogentisic acid, had no effect on tocopherol production. Homogentisic acid (HGA), however, was slightly stimulatory for the production of α-tocopherol. By contrast, phytol, a potential precursor of the phytyl side-chain, had a remarkable stimulatory effect, while geranylgeraniol was ineffective. It was particularly interesting that not only α- and β-tocopherols, the normal tocopherol constituents of safflower cells, but also γ- and δ-tocopherols were produced in the callus upon administration of phytol.

Table 1. Growth ratio and tocopherol production of safflower calli cultured on various media

Medium	Basal	Growth ratio	Tocopherol content (mg/100 g dry wt) α	β	Total
D 1 K 0.1*	MS	8.85	0.75	0.40	1.15
Basal	MS	8.21	1.60	0.36	1.96
IAA 1 K 0.1	MS	4.44	0.55	0.31	0.86
IBA 1 K 0.1	MS	6.10	0.57	0.16	0.73
NAA 1 K 0.1	MS	8.31	0.46	0.38	0.84
IAA 5 K 0.1	MS	5.82	1.60	0.45	2.05
K 1	MS	8.13	0.43	0.21	0.64
BA 1 (white)†	MS	13.57	1.10	0.16	1.26
BA 1 (yellow)	MS	9.53	0.54	0.36	0.90
D 1 K 0.1 CA 0.1%	RT‡	8.51	2.68	1.63	4.31
IBA 2 K 0.1 CA 0.1% (white)	RT	10.47	1.83	0.54	2.37
IBA 2 K 0.1 CA 0.1% (yellow)	RT	17.50	3.80	1.15	4.95

The growth ratio was determined by the increase of the fresh wt after 3 weeks culture of 1 g inoculum in 40 ml medium. The values are the quotient of the weight after 3 weeks culture and that of the inoculum.

Abbreviations used are as follows: D, 2,4-dichlorophenoxyacetic acid; K, kinetin; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, 1-naphthylacetic acid; BA, 6-benzyladenine; CA, casamino acid; MS, Murashige and Skoog's basal medium; RT, revised tobacco basal medium.

*The numbers shows ppm of plant growth regulators, except 0.1% in the case of casamino acid.

†Colour of the callus strain.

‡RT medium contains 0.55% inositol in this experiment, while MS medium contains 0.01%.

Finally, 50 and 100 ppm of phytol and homogentisic acid were administered singly or in combination according to the same method as above, i.e. administration on day 7 and incubation for 14 days (Table 3, Experiment No. 2), or subjected to short time biotransformation, i.e. administration on day 18 and incubation for three days (Experiment No. 1). The results showed that homogentisic acid was slightly effective in combination with phytol, except at the level of 100 ppm. In the long-term-biotransformation experiments, the optimal concentration (100 ppm) of phytol gave the highest level of tocopherol production, whereas when it was administered in combination with homogentisic acid tocopherol production was inhibited in spite of the lack of effect on growth.

Accumulation and metabolism of tocopherols in safflower cells

Aliquots of α-, γ- and δ-tocopherols were administered to cell suspension cultures. After culturing for two weeks, the cells and media were separately extracted with *n*-hexane, and the amount of each tocopherol was determined (Table 4). Almost no tocopherols were found in the medium. δ-Tocopherol was recovered in large quantities from the cells of the cultures fed δ-tocopherol, thus indicating that almost all of the tocopherols administered *in vitro* were incorporated into the cells. However, both α- and γ-tocopherols were rapidly metabolized and never accumulated in the cells. It is noteworthy that the level of β-tocopherol was increased in the cells of the cultures fed δ-tocopherol. The presence of S-adenosylmethionine: γ-tocopherol methyltransferase in the cells, was confirmed by an enzyme experiment carried out according to the method of ref. [14]. This enzyme showed low specificity to α-, β- and δ-tocopherol as compared with γ-tocopherol. These results strongly suggest that the biosynthetic route from γ- to α-tocopherol may be the main route of α-tocopherol biosynthesis and that from δ- via β- to α-tocopherol may be a minor one.

Identification of tocopherols

Each tocopherol (α-, β-, γ- and δ-) was identified by TLC using co-chromatography, and by HPLC using co-injection. Further confirmation of the identity of the α-tocopherol, the main product of the safflower cells, was provided by means of mass spectrometry of a HPLC-purified sample of α-tocopherol. The dominant feature was the appearance of an intense peaks at *m/z* 430 (M^+, 100%), 205 (12) and 165 (92), which are considered to be due to the characteristic fragmentation ions of tocopherol molecules and were consistent with those of the standard [15].

DISCUSSION

The cultures used in this study were descended from a callus prepared in June, 1976 from a flower bud of safflower. The highest growth and highest production of tocopherol was obtained with calli selected for growth on Revised tobacco basal medium supplemented with IBA (2 ppm), kinetin (0.1 ppm) and 0.1% casamino acid. This strain accumulated considerable amounts of tocopherols, mainly α-tocopherol (5–13 mg/100 g dry wt,) and grew very rapidly (the best growth ratio was × 17.5 in 3 weeks).

A time course of growth and tocopherol production by this strain (Fig. 2) showed that maximal tocopherol production was reached 25 days, i.e. on the 5th day after the highest growth ratio was obtained. This pattern is characteristic of secondary metabolites rather than primary ones, although tocopherol is generally thought of as a primary metabolite. This result is, however, in agreement with the observations that tocopherol is mainly stored in the seed of plants and that young fast-growing plants have very little α-tocopherol whereas slow-growing plants contain quite large amounts [9].

The enhancement of tocopherol production by administration of potential precursors of the tocopherol molecule was investigated by incubating the test compounds with one-week-old cell suspension cultures for a period of two weeks. The precursors tested (shikimic acid, tyrosine, homogentisic acid, geranylgeraniol, phytol and others) were dissolved in ethanol, methanol, chloroform, DMSO (dimethyl-sulphoxide), methyl cellosolve (ethylene glycol monomethylether) or Tween 80 (polyoxyethylene sorbitan monooleate) and then administered to the cell suspension cultures under sterile conditions. However, the growth of the cells was inhibited by almost all the solvents except Tween 80. Accordingly, Tween 80 was used as the solvent, but as a 20% solution, because

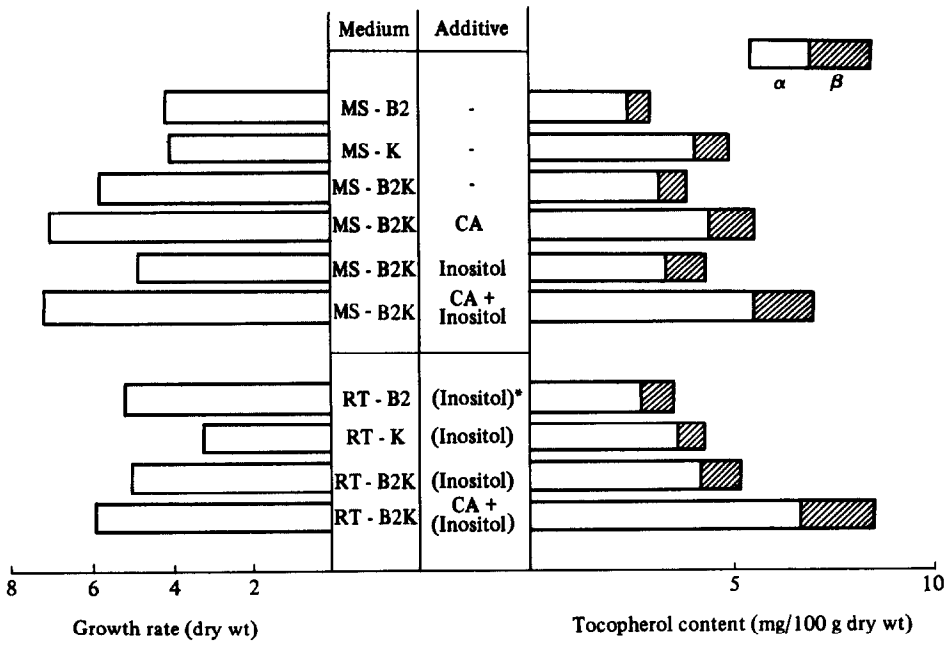


Fig. 1. Effects of plant growth regulators (1BA, 2 ppm; K, 0.1 ppm) and other additives contained in (CA, 0.1 %) B2KC medium on growth and tocopherol production of safflower suspension cultures. *RT medium contains 0.55 % inositol in this experiment, although the amount in MS medium is 0.01 %. Abbreviations are the same as Table 1, except B (indole-3-butyric acid).

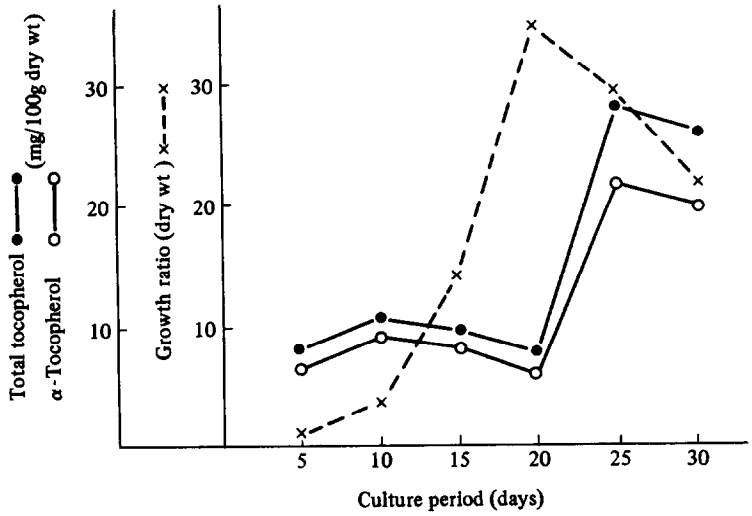


Fig. 2. Time course of growth and tocopherol production of safflower cells grown in suspension culture on B2KC medium. Growth ratio was determined the increase in the dry weight (lyophilized) of the cells after culturing 4 g of callus in 250 ml medium in a 1 l flask for the time shown. The values are the quotient of the weight after each time of culture and that of the inoculum.

undiluted Tween was very difficult to filter through the Millex-GS filter (Millipore Co., Ltd.) used for the administration of precursors. The results showed that safflower calli biosynthesize tocopherols by the tocopherol pathway, and not the tocotrienol pathway. Thus phytol, a potential side-chain precursor, increased the tocopherol content of the cells (62 mg/100 g dry wt as the total amount), whereas geranylgeraniol was without effect. Although the callus produced α-tocopherol as the major component and β-tocopherol as the minor, the administration of phytol led to the accumulation of α-, β-, γ- and δ-tocopherol. These facts suggest that the biosynthesis of α-tocopherol proceeds actively by way of γ- and/or δ-tocopherol in the presence of added phytol. As geranylgeraniol, a potential precursor of phytyl pyrophosphate needed for tocopherol biosynthesis, had no effect on

Table 2. Effects of various precursors on tocopherol production by safflower cultured cells

Precursor	Growth ratio	Tocopherol content (mg/100 g dry wt)				
		α	β	γ	δ	Total
Experiment No. 1						
No addition	8.53	14.83	0.44	1.23	—	16.50
p-Coumaric acid	7.05	15.38	1.92	—	—	17.30
D-Tyrosine	8.16	15.91	1.29	—	—	17.20
L-Tyrosine	8.37	16.15	1.49	—	—	17.64
L-Phenylalanine	7.91	15.04	1.41	—	—	16.45
p-Hydroxyphenyl-pyruvic acid	8.94	16.46	1.20	—	—	17.66
Homogentisic acid	9.35	17.93	1.21	0.32	—	19.46
Experiment No. 2						
No addition	5.90	8.27	0.82	—	0.10	9.19
Phytol	5.62	28.37	2.96	9.07	5.03	45.43
Isophytol	6.22	6.42	0.34	0.21	0.11	7.08
Geranylgeraniol	4.99	5.58	0.21	0.23	0.09	6.11

Each precursor, 100 ppm, was administered to a one-week-old culture which was subsequently incubated for a further 2 weeks. Experiments No. 1 and No. 2 were separately carried out under the same condition. All precursors were administered in 2 ml of 20% Tween 80 solution. The blank (no addition) was administered 2 ml of 20% Tween 80 solution.

Table 3. Effects of phytol and homogentisic acid (HGA) on tocopherol production by safflower cells

Precursor	Growth ratio	Tocopherol content (mg/100 g dry wt)				
		α	β	γ	δ	Total
Experiment No. 1						
No addition	5.24	3.69	0.71	+	+	4.41
HGA (100)	6.81	3.11	0.51	0.07	0.12	3.81
Phytol (100)	5.64	7.87	0.97	1.63	1.33	11.80
HGA (100) + Phytol (100)	4.98	6.37	2.60	2.62	3.53	15.12
Experiment No. 2						
No addition	5.84	2.68	0.77	—	—	3.45
HGA (50)	4.73	4.66	0.85	0.17	0.20	5.88
Phytol (50)	4.28	9.67	0.74	3.97	1.41	15.79
HGA (50 + Phytol (50)	4.35	16.11	2.00	6.72	2.98	27.81
HGA (100)	5.66	9.78	1.32	0.29	0.10	11.49
Phytol (100)	4.97	28.75	4.90	13.28	16.66	63.59
HGA (100) + Phytol (100)	4.61	18.61	3.11	5.82	4.09	31.63

Each compound was administered to a 18-day-old culture which was subsequently incubated for a further 3 days (short time biotransformation) in Experiment No. 1, and to a 7-day-old culture which was subsequently incubated for a further 14 days (long time biotransformation) in Experiment No. 2. The number in the parentheses shows ppm of each precursor.

tocopherol biosynthesis, it is clear, therefore, that in safflower cells there is no direct route from geranylgeraniol via geranylgeranyl pyrophosphate to tocopherol, i.e. that is the tocotrienol pathway, and that the route from geranylgeraniol via phytol and/or geranylgeranyl pyrophosphate to phytyl pyrophosphate may be rate limiting [16, 17]. By contrast, the administration of precursors of the aromatic ring, such as phenylalanine, D- or L-tyrosine, *p*-coumaric acid and *p*-hydroxyphenylpyruvic acid [18–20], had no effect on tocopherol production, except in the case of homogentisic acid [21], which had a slight stimulatory effect. Homogentisic acid is a very effective precursor in tocopherol biosynthesis in broken chloroplast systems [22–24], but in the safflower cells it was only slightly effective. These results suggest that homogentisic acid, a hydrophilic compound, has difficulty in penetrat-

Table 4. Accumulation and metabolism of α-, γ- and δ-tocopherols in safflower cultured cells. 100 ppm of each tocopherol was administered to a 1-week-old culture which was then incubated for a further period of 2 weeks

		Tocopherol content (mg/100 g dry wt)				
Tocopherol	Growth ratio	α	β	γ	δ	Total
No addition	7.59	4.58	0.76	—	0.09	5.43
α	7.48	10.89	0.73	—	0.13	11.75
γ	8.74	5.40	1.08	2.31	0.21	9.00
δ	7.46	10.23	10.23	0.84	165.17	183.70

ing the cytoplasmic membrane, compared with phytol, a hydrophobic compound. Furthermore, homogentisic acid is rapidly oxidized probably before being incorporated into the cell. This was evident from the fact that the cells and medium changed to a brown colour after administering homogentisic acid to the cell suspension. Moreover, as shown in the administration experiment in combination with phytol, both the high concentration of homogentisic acid and its long term biotransformation were inhibitory. The reasons for these effects are unknown. Further experiments are required in which homogentisic acid is administered to a cell-free system in the presence of an antioxidant reagent, such as ascorbic acid.

Soll and Schultz [22] believe that the product of the condensation of homogentisic acid and phytyl pyrophosphate, 2-demethylphytyl-plastoquinol in Scheme 1, is methylated to form phytylplastoquinol, which is cyclized to γ-tocopherol which is then methylated to produce α-tocopherol. δ-Tocopherol, which is sometimes found and is thought to be formed as a result of a side reaction, can be methylated to form β-tocopherol, and then α-tocopherol. Marshall *et al.*, [25] arrived at the same conclusions in their re-examination of tocopherol biosynthesis. The results presented here and the existence of *S*-adenosylmethionine: γ-tocopherol methyltransferase in Safflower cells (unpublished data) supports strongly the theory of Soll *et al.* Alpha- and γ-tocopherols were rapidly metabolized and were never accumulated in the cells at levels higher than the maximum pool size, which must vary with the age of the cells. Therefore, to bring about an increase in the α-tocopherol content of the cells, it will be necessary to enlarge the pool size in the cell. It is known that tocopherols are biosynthesized both inside and outside the chloroplast [26]. In fact, the tocopherol content in plant leaves is much higher in green tissue than in yellow tissue [9]. Therefore, the greening-up of cells may be a prerequisite for an increase of tocopherol content. The safflower cells used in this experiment never became green under illumination. So, if a chloroplast could be formed by any method, i.e. such as selection or mutation, an increase in the tocopherol production of the safflower cells would be expected. We are now in the process of isolating a green calli by a selection and mutation method.

EXPERIMENTAL

Cell culture. Flower buds of *Carthamus tinctorius* L., collected at Tokyo Metropolitan Medicinal Plant Garden in June 1976, were sterilized by treatment with 70% EtOH for 1 min and a 10% soln of bleaching powder for 5–10 min. The bracts were removed from the sterilized buds and the immature petals were obtained. The petals were washed with sterilized distilled H_2O (×2) and then cut into small pieces *ca* 5 mm in length. The pieces were then placed on Murashige and Skoog's basal agar medium [27] containing 1.0 ppm 2,4-dichlorophenoxyacetic acid (D), 0.1 ppm kinetin (K), (abbreviated as DK medium), 3% sucrose and 0.9% agar in a 50 ml Erlenmeyer flask. The flask was then left in the dark at 26° for 3–4 weeks. The calli (induced at a rate of about 95%) were maintained by subculture of 3-week intervals.

A part of the callus was isolated and inoculated into fresh media containing various plant growth regulators and media additives as follows. DKP and CM media are the DK medium supplemented with 1 ppm of *N*-phenyl-*N'*-(4-pyridyl)urea (P) and 7% coconut milk (CM), respectively. IK, BK and NK media are supplemented with 1 ppm of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthylacetic acid (NAA), respectively, in place of 2,4-D in the DK medium. K 1 and BA 1 media contained 1 ppm of K and 6-benzyladenine (BA) as a growth regulator. RC and B2KC media are Revised tobacco medium (RT) [28], modified by the addition of 0.55% inositol. RC medium contained 0.1% casamino acid (CA), 1 ppm D and 0.1 ppm K, and B2KC contained 0.1% casamino acid, 2 ppm IBA and 0.1 ppm K. The calli were subcultured at 3-week intervals.

Growth of callus. This was carried out on solid medium (40 ml) contained in a 100 ml Erlenmeyer flask or in shake culture in a liquid medium (250 ml) contained in a 1 l Erlenmeyer flask. The composition of the liquid medium was identical with the solid medium, apart from the omission of agar. The growth ratio was determined by the increase of the fresh and/or dry weight after 3 weeks culture. The values are the quotient of the weight after 3 weeks culture and that of the inoculum. Cell suspension cultures were started by inoculating callus tissue (10–20 g) into 250 ml liquid media, which was then shaken on a reciprocating shaker at 78 spm in the dark. Jar fermentor culture was carried out as follows. Static or liquid cultured cells, 100–200 g, were inoculated into 4 l medium contained in the 5 l vessel of a jar fermentor (Model MD-300, Marubishi Laboratory Equipment Co., Ltd.) and then cultured at 50 rpm with aeration (2 l/min) in the dark.

Extraction and quantitative analysis of tocopherols. The callus was collected by filtration, freeze-dried and crushed. The resulting powder was extracted with *n*-hexane (×2) under reflux for 8 hr in a stream of N_2 in the dark, and the extract was sepd from the residue by filtration. The filtrate was evapd *in vacuo* and then subjected to HPLC [29–31] on a Zorbax sil column (4.6 × 250 mm, Shimadzu) eluted with *n*-hexane–dioxane–EtOH (97.6:2.0:0.4) at a flow-rate of 1.5 ml/min. The fluorescence intensity of the column eluent was continuously monitored by using a Shimadzu RF-530 fluorescence spectromonitor. The

excitation wavelength (Ex) was set at 289 nm and detection was carried out at 325 nm. The R_t (min) of each tocopherol was as follows: α-tocopherol, 5.3; β-, 7.9; γ-, 8.6; δ-, 12.2. The content of each component was determined by means of a standard calibration curves. Each value in the cultured tissues shows the average of duplicate estimations in pairs of flasks of 2 or 3 different cultures.

Administration of biosynthetic precursors. 12.5 mg and 25.0 mg of the precursors to be tested was dissolved in 2 ml 20% Tween 80 soln, filtered through a Millex-GS filter (Millipore Co., Ltd) and administered to 250 ml of a 1-week-old cell suspension culture. The cell cultures were allowed to grow for 2 weeks and sepd into cells and medium by filtration. Administration of α-, γ- and δ-tocopherols was also carried out as above. Cells were lyophilized immediately after harvest and then extracted with *n*-hexane as described above. The medium was extracted with *n*-hexane. The amounts of tocopherols in each extract were determined by HPLC as above.

Chemicals. Tocopherols (α-, β-, γ- and δ-) used for calibration, identification and administration were purchased from Wako Pure Chemical Industries, Ltd. Phytol, isophytol and geranylgeraniol were gifts from Kuraray Co., Ltd. Others were commercial products.

Isolation and identification of α-tocopherol. The *n*-hexane extract (566 mg) from jar fermentor cultured cells (30.3 g dry wt on the DK medium) was chromatographed on a silica gel column (30 g Kiesel gel 60) and eluted with *n*-hexane–dioxane–EtOH (97.6:2:0.4). The eluate was collected in 50 ml fractions. α-Tocopherol (3.11 mg), present in factions 4 and 5, was purified by HPLC. The α-tocopherol was identified by TLC (co-chromatography), HPLC (co-injection method) and MS spectrometry. The operating condition was as follows: TLC: Kieselgel 60 F254, *n*-hexane–EtOAc (9:1; 0.28). HPLC was carried out according to the same method as described above. Mass spectra were obtained on a JEOL JMS DX-300 instrument. The mass spectrum of α-tocopherol showed as major fragments *m/z* 430 $[M]^+$ (100%), 205 (12) and 165 (92).

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