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Phenolic Substances in Tissue Cultures of *Centaurium erythraea*

L. MERAVÝ

Institute of Experimental Botany, Czechoslovak Academy of Sciences,
Na Karlovce 1, 160 00 Praha 6, Czechoslovakia

Abstract. Phenolic compounds 1,2,3-trihydroxy-5-methoxyxanthone, 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone, and 1,8-dihydroxy-2,3,4,6-tetramethoxyxanthone predominate in the callus tissue of *Centaurium erythraea*, their content increasing with culture age. By contrast, the contents of the derivatives of cinnamic, chlorogenic, and ferulic acids decrease or do not change. In the cell suspension culture of *C. erythraea* a larger amount of xanthones is synthesized than in the callus from which the suspension culture was derived. The content of phenolic acids is lower in the suspension culture than in the callus, but a larger number of low-molecular-mass phenolic substances occurs in the suspension culture than in the callus tissue.

Detailed understanding of processes taking place during the transition of plant tissues to tissue and cell cultures is a prerequisite for industrial and commercial utilization of plant tissue cultures for the production of natural substances. It is namely known that metabolism often changes in explant cultures due to the response of plant cells to different living conditions, which can manifest itself in the final balance of the synthesized secondary substances. Changes in the amount of a synthesized product can occur, which may in extreme cases result in its total disappearance (LEE *et al.* 1972), or in the formation of a completely new product (SLABECKA-SZWEYKOWSKA 1952, FORREST 1969). These responses can be assumed to be the consequence of the totipotency of the plant cell, whose inherited complete biochemical potential corresponding to the genome of the entire plant is prerequisite to certain biochemical diversity, the immediate expression of the genetic information being determined by the interaction of the cell with both endogenous and exogenous conditions (AITCHINSON *et al.* 1977). Thus the production of secondary metabolites in cell cultures can be regarded as a result of the differentiation process controlled by genetic factors together with environmental conditions (KURZ 1984). Different biosynthetic sequences, when compared to the original tissue, may occur in the cells in the culture either in the form of a newly synthesized compound or as partial or complete inhibition of the synthesis of some hitherto synthesized product. The inability of the culture to accumulate certain substance is not the result of the loss

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of the biochemical potential, it only indicates the impossibility of its realization under the particular conditions (LIAU and IBRAHIM 1973). When analyzing secondary substances synthesized by a culture one must not forget that the composition of their pool changes in the course of culture growth, especially depending on its biochemical and cytochemical differentiation (KING and STREET 1977), and that it is influenced by the inoculum size, medium composition, growth regulators and also by illumination (DAVIES 1972).

The above characteristics also fully apply to phenolic substances which form the most numerous group of secondary metabolites. A number of tissue cultures has been characterized with exactly defined parameters of the biosynthesis of phenolic substances (*e.g.* SAHAI and SHULER 1984). From their comparison it follows that each tissue and cell culture is specific to a considerable degree, and the action of particular cultivation factors cannot be applied without verification to a culture derived from a different plant source.

In this paper results obtained during isolation and identification of phenolic substances from the callus and the suspension cultures of *Centaurium erythraea* are summarized with the aim of their mutual comparison.

MATERIAL AND METHODS

Tissue Cultures

Callus cultures (strain C 9) derived from roots of *Centaurium erythraea* RAFN by Dr. Barešová from the Institute of Experimental Botany (BAREŠOVÁ and KAMÍNEK 1984) were cultivated at 25 °C under natural light regime on LS medium (LINSMAIER and SKOOG 1965) with the addition of 2,4 D (1 mg l^{-1}). Cell suspension cultures derived from 28-day-old caluses of *C. erythraea* were maintained under the same conditions and on the same medium as the calluses. The cultures, 30 ml in 250 ml flasks, were cultivated on a rotary shaker. Samples for analyses were taken from the third subculture, with the subcultivation interval of 21 days.

Extraction of Phenolic Substances

Calluses were used directly for extraction, cells from suspension cultures were first separated from the medium by vacuum filtration, washed with distilled H_2O, 1 min dried with air stream, and then fresh matter determined. Samples with fresh matter of 10 g were extracted with 3 × 20 ml of 90 % ethanol (2 min at boiling point, 1 h at laboratory temperature followed by final washing). Combined extracts were evaporated in vacuum to a final volume of 3 ml.

Analysis of Phenolic Substances

Phenolic substances present in the extract were separated by means of paper chromatography as earlier (MERAVÝ 1985). Besides the R_F values, UV detection methods including the application of gaseous ammonia, and recording of UV spectra after the application of agents causing specific spectral shifts (NaOAc, $AlCl_3$, $AlCl_3$ + HCl, see MABRY *et al.* 1970) were employed for the identification of phenolic substances. The spectra of meth-

TABLE I

Spectral properties of phenolic compounds isolated from callus and cell cultures of *Centaurium erythraea*

No.	Compound	Reagent	λ max. [nm]
1	1,2,3-trihydroxy-5--methoxyxanthone	MeOH	244, 259, 274″, 315, 366
		NaOAc	240, 261″, 293, 350, 418
		$AlCl_3$	245, 266, 282, 345, 418
		$AlCl_3$/HCl	245, 266, 281, 338, 420
2	1-hydroxy-3,5,6,7,8-penta-methoxyxanthone	MeOH	256, 314, 361
		NaOAc	247, 257, 274″, 317
		$AlCl_3$	260. 274″, 321, 345″, 418″
		$AlCl_3$/HCl	260, 274″, 323, 345″, 418″
3	1,8-dihydroxy-2,3,4,6-te-tramethoxyxanthone	MeOH	235, 256, 276″, 334
		NaOAc	256, 276″, 334
		$AlCl_3$	235, 276, 373
		$AlCl_3$/HCl	235, 276, 373
4	Sakuranin	MeOH	280, 317″
		NaOAc	279, 314″
		$AlCl_3$	280, 313″
		$AlCl_3$/HCl	280, 310″
5	Isoflavone	MeOH	267, 330
		$AlCl_3$	267, 332
		$AlCl_3$/HCl	266, 332
6	Chlorogenic acid	MeOH	242, 305″, 330
		NaOAc	242, 300″, 327
		$AlCl_3$	242, 305″, 330
		$AlCl_3$/HCl	242, 305″, 330
7	Ferulic acid conj.	MeOH	288, 321
		NaOAc	286, 320
		$AlCl_3$	285″, 331
		$AlCl_3$/HCl	286″, 332
8	Derivative of cinnamic acid I	MeOH	278, 311
		NaOAc	274, 310
		$AlCl_3$	270, 330
		$AlCl_3$/HCl	270, 338
9	Derivative of cinnamic acid II	MeOH	247, 314
		$AlCl_3$	247, 264″, 322
		$AlCl_3$/HCl	247, 322

The symbol ″ indicates inflexion or shoulder

anol eluates of chromatographic spots were recorded on a spectrophotometer Specord UV VIS (Carl Zeiss, Jena, GDR); the readings obtained also served for the determination of the amounts of phenolics. Identical or structurally related compounds were used as standards for this purpose. Every experiment was replicated three times.

RESULTS

Phenolic Substances in Callus Cultures

The same phenolic substances, although in different amounts, were identified in the extracts of 28-day-old and 51-day-old calluses, respectively. The dominant group was represented by three xanthones, and besides them two phenolic acids and the flavanone sakuranin occurred in the extracts (Table 1). Substances *1* and *3* were identified according to their spectral

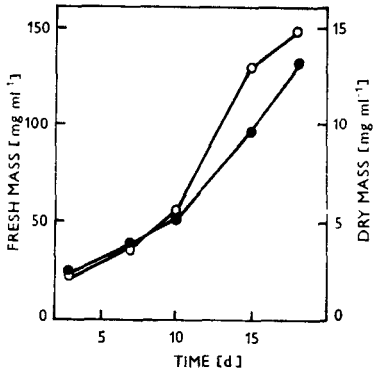

Fig. 1. Growth curve of the cell suspension culture of *Centaurium erythraea*.

properties consistent with literature data as 1,2,3-trihydroxy-5-methoxyxanthone (NESHTA *et al.* 1983a) and 1,8-dihydroxy-2,3,4,6-tetramethoxyxanthone (TAKAGI and YAMAKI 1982). Substance *2* was identified by comparison with the standard compound as 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone. Substance *6* was similar in its mobility to chlorogenic acid, and its spectral properties (especially the negative reaction with $AlCl_3$) indicate that it is chlorogenic acid whose o-positions are substituted with hydroxyl groups. Substance *9* resembled hydroxycinnamic acids as far as fluorescence in UV light and spectral properties are concerned; the derivative was not identified in detail.

Phenolic Substances in the Cell Suspension Culture

All the above mentioned substances with the exception of the derivative of cinnamic acid (*9*) were identified in the extract of 18-day-old suspension culture (at the beginning of the transition to the stationary phase — Fig. 1); in addition four other phenolics were detected. Substance *4* corresponds in its R_F values and spectral properties to the flavanone sakuranin. According to its spectral properties substance *5* can be considered an isoflavone, the final identification of it was not successful. Substance *7* is an ester of ferulic acid which was identified after alkaline hydrolysis by comparison with the standard compound. And finally, substance *8* was also not identified precisely; it could only be characterized as a derivative of cinnamic acid.

Differences in the amounts of particular phenolic substances occurred in callus cultures of different age (Table 2), which indicated considerable fluc-

TABLE 2

The content of phenolic compounds in tissue cultures of *Centaurium erythraea* (μg g^{-1} fresh matter). The values are means of three determinations

No.	Compound	Callus		Suspension
		28 d	51 d	18 d
1	1,2,3-trihydroxy-5-methoxy-xanthone	17	23	196
2	1-hydroxy-3,5,6,7,8-penta-methoxyxanthone	34	72	224
3	1,8-dihydroxy-2,3,4,6-te-tramethoxyxanthone	traces	18	3
4	Sakuranin	traces	traces	15
5	Isoflavone	0	0	9
6	Chlorogenic acid subst.	78	traces	53
7	Ferulic acid conj.	0	0	4
8	Derivative of cinnamic acid I	0	0	3
9	Derivative of cinnamic acid II	10	7	0

tuation of the level of the phenolics during callus culture growth. The amount of xanthones increased with the age of the culture, whereas the content of phenolic acids decreased. Considerable differences were also recorded when callus and suspension cultures were compared. The two cultures were similar in the dominant occurrence of 1,2,3-trihydroxy-5-methoxyxanthone and 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone; a high content of the derivative of chlorogenic acid was also remarkable.

DISCUSSION

The finding that xanthones are characteristic and dominant phenolic substances of the genus *Centaurium* is in agreement with literature data. The occurrence of 1,2,3-trihydroxy-5-methoxyxanthone and 1,8-dihydroxy-2,3,4,6-tetramethoxyxanthone in the species *C. erythraea* was already reported (NESHTA *et al.* 1983a, TAKAGI and YAMAKI 1982), the occurrence of 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone was recorded in the species *C. littorale* (VAN DER SLUIS 1985). The occurrence of the flavanone sakuranin and of isoflavone in the genus *Centaurium* has not yet been reported. By contrast, 1,8-dihydroxy-3,5-dimethoxyxanthone, 1,4-dihydroxy-3,5-dimethoxyxanthone, 1,6,8-trihydroxy-3,5,7-trimethoxyxanthone, and 1,8-dihydroxy -3,4,6-trimethoxyxanthone which were identified in intact plants of *C. erythraea* (TAKAGI and YAMAKI 1982, NESHTA *et al.* 1982, 1983b, 1984) were

not detected in the analyzed samples of callus and cell suspension cultures. The abundance of xanthone synthesis in the genus *Centaurium* has also been demonstrated by the reported occurrence of five xanthones in *C. linarifolium* (PARRA *et al.* 1984a,b), three xanthones in *C. pulchellum* (MIANA and AL-HAZIMI 1984), four xanthones in *C. littorale*, three xanthones in *C. scilloides*, *C. tenuiflorum* and *C. chloodes*, and of two xanthones in *C. spicatum* (VAN DER SLUIS 1985, VAN DER SLUIS and LABADIE 1985).

Phenolic acids and their derivatives represent metabolites whose occurrence in plant tissues is quite common, but distinct differences between the callus and the suspension cultures exist even in their pool. The suspension cell culture synthesizes at the beginning of the transition to the stationary phase a larger number of phenylpropanoids than the callus culture. The amounts of most phenolics also were larger in the suspension culture, with a clear predominance of xanthones *1* and *2*. Higher content of some phenolic acids (*6* and *9*) was recorded only in vigorously growing callus cultures (28-day-old), and higher xanthone *3* content was found in 51-day-old callus cultures (on the boundary of viability — observed microscopically). This culture contained a generally higher content of xanthones and *vice versa* a lower amount of phenolic acids than the vigorously growing callus culture. Thus ageing of the callus and especially cultivation of cultures derived from it cause and initiate metabolic processes resulting in the accumulation of xanthones and flavonoids and a certain decrease in the content of phenolic acids. The several-fold increase in the content of xanthones *1* and *2* during 18 days of the third subculture, when the relative amount of xanthones from the total content of the phenolics studied was as high as 85 %, is especially conspicuous.

With regard to the same cultivation conditions, the differences in the phenolic metabolism are the consequence of a different expression of plant material under different forms of growth (callus — suspension). For example, differentiation of tissues with increased flavonoid synthesis may occur in the callus culture (BRUNET and IBRAHIM 1973). Vacuolization as a consequence of certain differentiation inside a growing culture can offer increased ability of accumulation of phenolic substances (AITCHINSON *et al.* 1977). A certain degree of differentiation can also be assumed to exist within cell aggregates in the cell suspension culture, resulting in subsequent changes in the metabolism of phenolic substances, especially lignin (KING and STREET 1977), and nonspecific effects on other phenolic metabolic pathways.

Thus it can be summarized that both the callus and suspension cultures synthesize different patterns of phenolic substances than intact plants and so does also each of them. Further details of these phenomena will be discussed in the following paper.

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