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Phenolic Substances in the Cell Suspension Culture of *Centaurium erythraea*

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Abstract. The content of phenolic substances in the cell suspension culture of *Centaurium erythraea* fluctuated during a 21-day-long subcultivation period in dependence on the growth phase. The total relative content of the phenolics reached its maximum at the time of transition to the exponential growth phase, similarly as the fraction of free phenolic acids, glycosides, and the fraction of phenolic acids released from the cells after alcaline hydrolysis. On the other hand, the content of phenolic acid esters decreased at this growth phase of the culture. Changes in the level of phenolic substances in the culture medium corresponded in their character to changes in the relative content of the phenolics in the cells.

In spite of extensive investigations of plant phenolic substances, many questions relating to the regulation of the metabolism of phenolics in intact plants remain unanswered. The extent of our knowledge of this subject in tissue and cell cultures is even more limited, although they certainly represent a rather simpler biological system. The dynamics of the biosynthesis of phenolic substances during the cultivation period is immediately influenced by cultivation conditions, especially by light, by medium composition, and by growth regulators (HAHLBROCK and WELLMANN 1970, CONSTABEL *et al.* 1971, DAVIES 1972, SAHAI and SHULER 1984, and others), which together with the genetically controlled individuality of each particular culture makes it considerably difficult to interpret and generalize the results obtained. Thus, the investigation of each culture must be conducted with regard to its particular specificity and characteristics and with full realization of the limited validity of the data obtained.

The analyses and characteristics of phenolic substances synthesized in the callus and suspension cultures of *Centaurium erythraea* were reported earlier (MERAVÝ 1987). In this paper changes in the content of phenolic substances occurring during one subcultivation of the cell suspension culture of *C. erythraea* are described and summarized. The fractionation method employed made it possible to differentiate the specific character of changes in particular categories of the phenolics.

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MATERIAL AND METHODS

The cell suspension culture of *Centaurium erythraea* RAFN derived from the callus was subcultivated at 21-day intervals under the same conditions as described earlier (MERAVÝ 1987).

Extraction and fractionation of phenolic substances: Cells from the suspension culture were separated from the medium by vacuum filtration, washed with distilled water, dried with air stream for 1 min, and then used for the determination of fresh and dry masses and for the extraction of phenolic substances. Cell samples of 5 g fresh mass were frozen in liquid nitrogen and, after thawing, extracted with three 16 ml portions of boiling 80 % methanol under a retro-cooler, always for 15 min. Combined extracts were fractionated using the method described by ZADERNOWSKI and KOZLOWSKA (1983) with some modifications. The solvent was evaporated at 50 °C under reduced atmospheric pressure and the solid remainder obtained was dissolved in 40 ml of H_2O, the pH value of which was adjusted with HCl to pH 2. After the extraction with 3×40 ml of diethylether, the ether extracts obtained were combined, water was removed from them with anhydrous Na_2SO_4, diethylether was evaporated, and the remainder representing the fraction F_1 (containing especially free phenolic acids, coumarins and flavonoid aglycones) was dissolved in 2 ml of 50 % ethanol. The acid aqueous solution remaining after the extraction with diethylether was neutralized with 2 M NaOH and then subjected to alkaline hydrolysis (for 4 h, under N_2 atmosphere, at laboratory temperature) after the addition of more sodium hydroxide to make its concentration to 2 M. The hydrolysate was acidified to pH 2 and again extracted with diethylether, then the solvent was evaporated and the remainder which represented the fraction F2 containing the originally esterically bound phenolic acids was dissolved in 2 ml of 80 % ethanol. The hydrolysate remaining after the extraction with diethylether was extracted with 3×40 ml of ethylacetate, combined ethylacetate extracts were evaporated, and the remainder which represented the fraction F3 containing above all glycosides was dissolved in 2 ml of ethanol. The cell homogenate remaining after initial extraction with methanol was dissolved in 40 ml of 2 M NaOH and hydrolyzed for 4 h at laboratory temperature under N_2 atmosphere. The hydrolysate obtained was filtrated and acidified with 6 M HCl to pH 2, extracted with ethylether and, using the above described procedure, transferred to the fraction F4 containing phenolic acids originally bound to cell structures and insoluble in 80 % methanol.

Phenolic substances contained in the culture medium were separated only to two fractions using a simplified extraction and fractionation procedure. Following the extraction and separation of the FM_1 fraction with diethylether, the acid aqueous solution was extracted with ethylacetate, which resulted in the separation of the fraction FM_2 containing bound phenolic substances.

The phenolic substances present in the fractions were determined with Folin-Denis reagent (ZAPROMETOV 1971), the absorbance of the reaction products was read at 730 nm using a SPECOL 20 spectrophotometer (Zeiss, GDR). The amount of the reaction products was expressed in values obtained by relating the absorbance of the fraction to the calibration curve obtained with standard gallic acid.

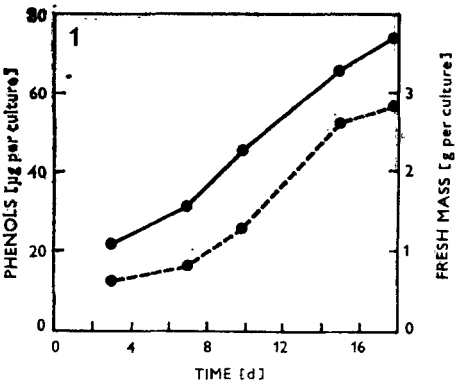

Fig. 1. Total absolute content of phenolic substances during cell culture growth. Symbols: Full line — the content of phenolic substances; dashed line — cell culture growth.

RESULTS AND DISCUSSION

The total content of phenolic compounds related to one culture increased with increasing dry matter yield (Fig. 1). This indicates that phenolic acids are synthesized in the cells of the suspension culture from the early stages of cell growth and that under the given cultivation conditions they are also synthesized during the stage of intensive cell division and cell growth. From the relative expression it follows that the content of phenolic compounds related to dry matter increases in the lag-phase of cell growth and reaches its maximum at the time of transition to the exponential phase of growth, followed by a gradual decrease which levels off at the onset of the stationary phase of growth (Fig. 2). Increased accumulation of phenolic substances at the time of transition to the exponential phase of growth is a matter that deserves attention. From the results of fractionation it follows (Figs. 3, 4) that the fraction of bound phenolic acids is not involved in this increased accumulation (the content of which shows a decreasing trend at this growth stage), and the cause of this phenomenon has not yet been established. The relationship between the contents of fractions F_1 and F_2 suggests that the changes in their content might be at least partially due to mutual transitions and exchanges between free and esterically bound phenolic acids. The relative increase in the content of both glycosides and phenolic acids bound in the fraction F_4 (obviously in the cell walls) shows that the increase in the content of phenolic substances at the time of the transition to the exponential phase

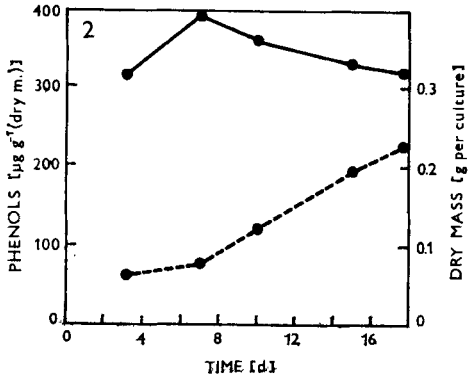

Fig. 2. Total relative content of phenolic substances during cell culture growth. Symbols: Full line — the content of phenolic substances; dashed line — cell culture growth.

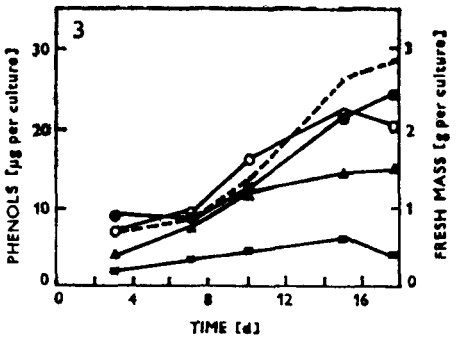

Fig. 3. Absolute content of phenolic substances in fractions during cell culture growth. Symbols: Dashed line — cell culture growth; ○ F_1; ● F_2; ▲ F_3; ■ F_4.

of growth is a rather general phenomenon which manifested itself in the content of most of the fractions under investigation. It also was found in tobacco callus cultures when the total amount of phenolics was determined (ZÁDOR *et al.* 1985) and in the cell suspension cultures of tobacco in cases of the same fractions as described in this paper (CVIKROVÁ and HRUBCOVÁ personal communication), and also in the cell suspension culture of *Vitis vinifera* when anthocyans were investigated (YAMAKAWA *et al.* 1983).

Considering the likely causes of the enhanced synthesis of phenolic substances before the onset of the exponential phase of growth, two mechanisms appear to be plausible for their explanation. One of them could explain the increasing content of phenolics in terms of the plain consequence of a certain excess in the content of substrates necessary for their biosynthesis at a time of a relatively low rate of their utilization in protein synthesis. The second mechanism may be apprehended in terms of a physiological response of the plant cells (to a certain extent) to stress conditions, which, as may be assumed, the cultivation in cell cultures represents when compared with the intact plant. The increase in the content of phenolics represents a characteristic consequence of plant reactions to some stress situation, as for example infestation by pathogens (FRIEND 1979, HATTORI and OHTA 1985), mechanical damage of plant tissues (KOJIMA and URITANI 1972, TANAKA and URITANI 1979), or UV radiation (BEGGS *et al.* 1985). The physiological nature of these reactions has not yet been explained and characterized in full detail, nor has that of the so-called "dilution effect", the involvement of which in the accumulation of the phenolics in the above mentioned growth phase of the subculture cannot be excluded. However, because the maximum in their

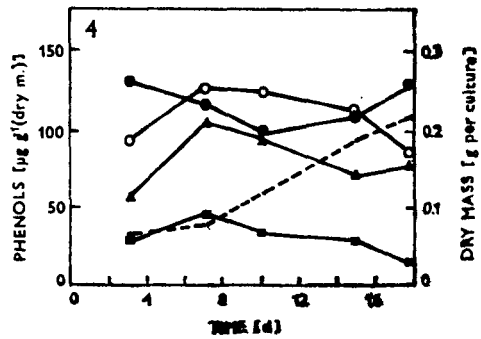

Fig. 4. Relative content of phenolic substances in fractions during cell culture growth. Symbols: Dashed line — cell culture growth; ○ F_1; ● F_2; ▲ F_3; ■ F_4.

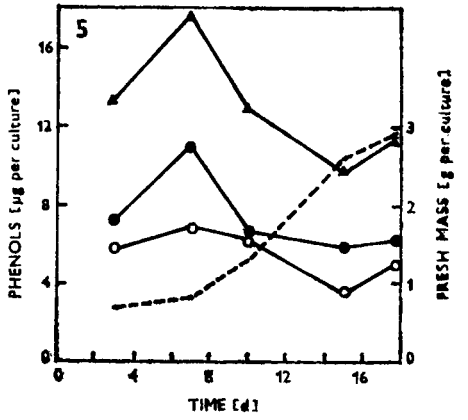

Fig. 5. Absolute content of phenolic substances in the cultivation medium. Symbols: Dashed line — cell culture growth; ○ FM_1; ● FM_2; ▲ total content of phenolics.

content was reached on day 7, that is as late as at the time of the transition to the exponential stage of growth, when the culture was virtually fully consolidated after the subcultivation and when intensive cell divison started, the involvement of this mechanism does not appear likely. Moreover, the hypothesis about a direct relationship between the induction of the enzymes involved in the pathway of the phenylpropane metabolism caused by the transfer of cells into fresh medium and the synthesis of cinnamate esters (HAHLBROCK *et al.* 1981) would confirm the above mentioned absence of the "dilution effect" in case of its general validity, because the level of esterically bound phenolic acids in the cells of the culture studied reached its maximum earlier than that of other phenolic fractions, and at the given growth phase it already showed a decreasing trend.

The decrease in the total level of phenolic substances during the late stage of the exponential growth stage of the culture could be explained in terms of a high rate of utilization of the substrates of the shikimate pathway — aromatic amino acids in protein synthesis at the phase of intensive cell division and cell growth. The decrease in the content of the fraction of bound phenolic acids suggests the possibility of the involvement of the system free

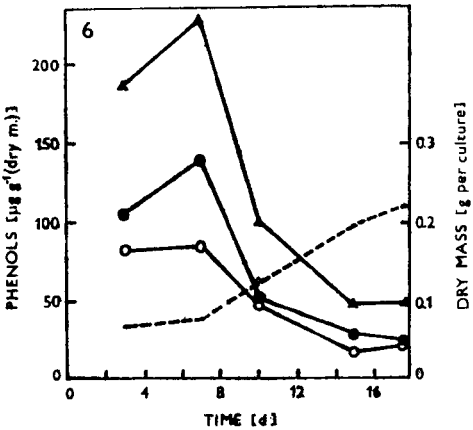

Fig. 6. Relative content of phenolic compounds in the cultivation medium. Symbols: Dashed line — cell culture growth; ○ FM_1; ● FM_2; ▲ total content of phenolics.

phenolic acid—bound phenolic acid during the growth cycle of the cell culture.

Phenolic substances synthesized by the cell suspension culture were to some extent released into the medium. Their content in the medium (Figs. 5, 6) which at its maximum amounted to nearly 60 % of the content in the cells, was not dependent on the total content of the phenolics in the culture, but on their relative content (related to dry matter unit). The maximum content of the phenolics in the medium which occurred at the time of transition to the exponential phase and the subsequent decrease in their content corresponded in its time course to changes in the relative content of phenolics in the cells of the culture. What is of some interest is the decrease in the absolute level of the phenolic substances in the medium to nearly one half of the maximal value during the exponential and linear phases of growth. Degradation of the phenolics by enzymes present in the medium (especially by peroxidases — BERLIN and BARZ 1975) is likely, although the possibility of their utilization at the stage of intensive cell division and growth cannot be excluded either.

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