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MEDIUM- AND LIGHT-INDUCED FORMATION OF SERPENTINE AND ANTHOCYANINS IN CELL SUSPENSION CULTURES OF *CATHARANTHUS ROSEUS*

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Key Word Index—*Catharanthus roseus*; Apocynaceae; tissue culture; light; medium; biosynthesis; indole alkaloids; anthocyanins.

Abstract—By irradiation with fluorescent light, medium-induced cell cultures of *Catharanthus roseus* accumulated anthocyanins and the indole alkaloid serpentine. The formation of both compounds was inhibited by phosphate and nitrogen-containing mineral salts and stimulated by high sucrose concentrations. The accumulation of serpentine was preceded by an increase and subsequent decrease of its biogenetic precursor ajmalicine, which was the predominant alkaloid of medium-induced cultures in the dark. High concentrations of serpentine or anthocyanins were observed only in a small proportion of all cells present in a medium-induced culture. The aglycones of the anthocyanins were identified as petunidin, malvidin and hirsutidin.

INTRODUCTION

In tissue cultures of *Catharanthus roseus* it has been shown that light has a stimulating effect on the accumulation of anthocyanins [1] and of the indole alkaloid serpentine [2, 3]. Recently, we reported on a medium-induced formation of ajmalicine and phenolic compounds in dark-grown cell suspension cultures of *Catharanthus roseus* [4]. In order to influence secondary metabolism further, medium-induced cells were kept under continuous illumination. Quantitative and qualitative changes of indole alkaloids and phenolic compounds under these conditions, especially the concomitant formation of serpentine and anthocyanins, are described here.

RESULTS

Formation of serpentine and anthocyanins

After transfer of 2-week-old cell suspension cultures of *Catharanthus roseus* grown in Murashige–Skoog (MS) medium [5] containing 2×10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D) into a 10-fold volume of an 8% aqueous sucrose solution a rapid increase of the intracellular level of the indole alkaloid ajmalicine was observed [4]. When the medium-induced cells were kept in the dark, ajmalicine was the predominant alkaloid formed after an incubation period of 2 weeks (Table 1). Upon continuous irradiation with fluorescent lamps preferably emitting blue light, these cells contained lower levels of ajmalicine and considerable amounts of its oxidation product serpentine were found. At the same time there was a characteristic increase of phenolics (Folin-positive material) and the cultures turned red due to the formation of anthocyanins (Table 1). These pigments were not found in dark-grown cultures which showed a pale yellowish colour. The formation of anthocyanins during illumination could be easily observed to occur 4–7 days after cell transfer. A similar product accumulation occurred after transfer into MS medium deficient of phosphate and nitrogen-containing mineral salts (Table 2). Growth in the induction media was similarly low in dark- and light-grown cultures.

Generally our product analysis was an end-point

Table 1. Influence of light on the medium-induced formation of secondary compounds in *Catharanthus roseus* cell suspensions

	Ajmalicine (mg/g fr. wt)	Serpentine (mg/g fr. wt)	Phenolics (E_{725}/g fr. wt)	Anthocyanins (μmol/g fr. wt)
Dark	0.56	0.02	16.8	0.001
Light	0.28	0.35	82.9	0.50

7 ml of a 2-week-old suspension culture (*ca* 1.7 g cell fr. wt) was transferred to 70 ml of an 8% aq. sucrose soln and incubated for another 2 weeks.

Table 2. Influence of various modified MS media [5] on illuminated *Catharanthus roseus* suspension cultures

Modification	Ajmalicine (mg/g fr. wt)	Serpentine (mg/g fr. wt)	Phenolics (E_{725}/g fr. wt)	Anthocyanins (μmol/g fr. wt)	Growth (g fr. wt/flask)
$+2\times10^{-6}$ M 2,4-D	0.01	0.01	27.5	0.06	15.0
None	0.23	0.04	20.0	0.04	6.37
−Pi	0.05	0.03	31.3	0.05	1.8
−Inorganic N	0.17	0.13	34.9	0.05	3.8
−Pi − inorganic N	0.24	0.35	70.0	0.48	2.1

Basal medium = MS medium +8% sucrose; cell transfer as described in Table 1.

determination after 2 weeks of incubation. To get more information on the dynamics of product accumulation a time-course of the various compounds was monitored (Fig. 1). After cell transfer into an 8% aqueous sucrose solution a rapid increase of ajmalicine and anthocyanins was observed during the first week of illumination. Later on the ajmalicine content decreased again whereas progressively increasing levels of serpentine were measured. At the end of the incubation period the cells contained similar amounts of both alkaloids.

The products were accumulated within the vacuolar space of the cells, as seen by microscopy in visible light (anthocyanins) and by fluorescence (serpentine), whereas no significant amounts were found in the medium. Apart from the major products ajmalicine and serpentine, a large number of other, unidentified alkaloids could be detected by TLC.

Microscopy, however, revealed that the culture was highly heterogeneous with regard to the accumulation of anthocyanins and serpentine. After medium-induction with concomitant irradiation, only few cells (*ca* 5%) showed a high content of anthocyanins, whereas the majority had only a slight or no red colour. A similar situation was observed for the distribution of serpentine. Furthermore, cells containing serpentine but no anthocyanins could be observed. Due to quenching of fluorescence by anthocyanins, we could not judge the serpentine content of anthocyanin-containing cells. The mean value for the intracellular concentration of anthocyanins was calculated as 0.6 mM from the absorption of the MeOH–HCl extract from 0.5 g (fr. wt) cell material using an ϵ of 33000 [6]. By direct microphotometric determination of a single, high producing cell a value of 2.8 mM was estimated. For calculating the anthocyanin concentration in aqueous solution, the ϵ_{530} of malvin chloride was determined in 0.01 N HCl as 11000 and the cell diameter was measured to be 45 μm.

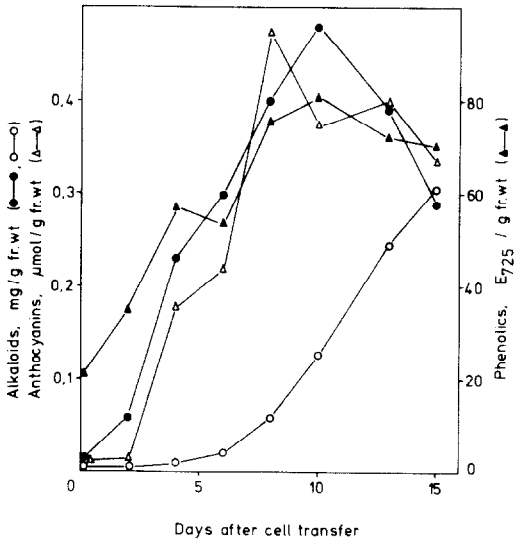

Fig. 1. Time course of the accumulation of indole alkaloids (ajmalicine ●—● and serpentine ○—○) and phenolic compounds (total phenolics ▲—▲ and anthocyanins △—△) in cell suspension cultures of *Catharanthus roseus* after cell transfer into 8% sucrose solution and continuous irradiation with fluorescent light.

Identification of the anthocyanidins

After hydrolysis the anthocyanidins could be separated from MeOH extracts of medium-induced, illuminated cultures by PC in solvent E (see Experimental). Two of these components were identified by co-TLC on cellulose in solvents E, F, and G with the references malvidin and petunidin. This result was confirmed by HPLC analysis. Hydrolysates of the MeOH–HCl extracts from cell cultures and intact petals of *Catharanthus roseus* showed identical separation patterns on HPLC and TLC. Therefore, and from R_f values and spectral data, the third anthocyanidin was found to be hirsutidin. Thus under our conditions petunidin, malvidin and hirsutidin are formed by *Catharanthus* cell suspensions. The same anthocyanidins were found in callus tissue [1] and in intact flowers [7] of this plant. The relative amounts, however, differed in flowers and cell suspensions as seen by HPLC analysis. Hirsutidin was the most prominent pigment in petals, whereas the extracts from cell suspensions contained mainly the less methylated compounds petunidin and malvidin.

Influence of minerals and sucrose

Recently we have demonstrated that in dark-grown cultures the medium-induced formation of secondary compounds is very sensitive to phosphate and nitrogen-containing minerals [4]. When these nutrients were present in concentrations commonly used for plant cell culture the accumulation of alkaloids and phenolics was largely reduced. Effects of modified MS media [5] on illuminated *Catharanthus* cultures are shown in Table 2. Highest accumulation of alkaloids, anthocyanins and total phenolics were

again observed in the absence of phosphate and mineral nitrogen. The addition of phosphate or KNO_3 and NH_4NO_3 inhibited the anthocyanin accumulation by 90% whereas the alkaloid level was reduced by 85% under the influence of KNO_3 and NH_4NO_3 and by 48% by the addition of phosphate. Thus, the inhibition caused by these minerals, especially by phosphate, was lower under light conditions than in the dark [4]. When both phosphate and mineral nitrogen were present (complete MS medium) similar amounts of ajmalicine to those occurring in their absence but very low levels of serpentine were found; thus, the sum of both alkaloids was reduced by 54%. The accumulation of alkaloids and phenolic compounds in the dark was further dependent on the sucrose concentration, showing a maximum at 8% [4].

When the cells were transferred to various sucrose solutions and incubated in the light, distinct effects on the quantity and the quality of the products were observed (Fig. 2). Raising the sucrose concentration from 2 to 11% caused a parallel increase of serpentine and anthocyanins by more than 300%, whereas the ajmalicine proportion of the sum of ajmalicine and serpentine fell from 66 to 26%. Concomitantly the content of total phenolic compounds increased only by 34%. Thus, a slight increase of the overall content of secondary compounds but a strong shift of the qualitative product pattern occurred on changing the sucrose concentration.

Altering the sucrose concentration raised the interesting question of the optimal reference basis for values of product levels. Clearly, the cell as a biological unit would be the most suitable basis to which these data should be referred. However, cell counting is rather laborious and time consuming. Therefore most of the published data are referred to either fr. or dry wt. We observed that not only during a growth cycle in MS medium supplemented with 2×10^{-6} M 2,4-D but also upon variation of the sucrose concentration, the cell number of *Catharanthus* cells showed a closer correlation with fr. than dry wt (K. H. Knobloch, unpublished data). This fact was attributed to a high variability of cell dry wt caused presumably by varying concentrations of vacuolar solutes which, in turn, are influenced by the nutritional environment. For optimal comparison of cells in various media, therefore, product levels were referred to cell fr. wt.

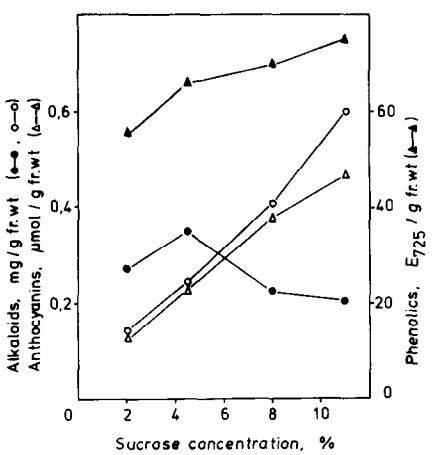

Fig. 2. Influence of the sucrose concentration on the accumulation of ajmalicine (●—●), serpentine (○—○), total phenolics (▲—▲) and anthocyanins (△—△). Cells were transferred to the indicated sucrose solutions and incubated under continuous illumination for 2 weeks.

DISCUSSION

Our results give further support to the idea that environmental conditions are important factors in the regulation of secondary metabolism in cell cultures and that also non-selected cells can have a high potential for product accumulation.

For *Catharanthus roseus* cell cultures, the influence of phytohormones and other medium components has been studied [3, 8]. Recently we demonstrated that very basic nutrients such as nitrogen-containing mineral salts and phosphate are inhibitory to the formation of alkaloids and phenolics in dark-grown cultures [4]. As a result, modified media either deficient of both components or containing only sucrose were used to induce secondary metabolism. Furthermore, regulation of the formation of cinnamoylputrescines in cell cultures of *Nicotiana tabacum* has been recently found to be phosphate-dependent [9].

Similar effects of phosphate on secondary metabolism of micro-organisms have been reported [10]. The general increase of secondary compounds under medium conditions which restrict growth (low auxin, phosphate and nitrogen) is a strong argument for an antagonistic regulation of growth and secondary metabolism as discussed earlier in terms of competition for the amino acids as common precursors for protein synthesis and secondary metabolism [11]. The findings, however, that the medium-induced formation of secondary compounds in *Nicotiana* and *Catharanthus* could be correlated with enhanced activities of phenylalanine ammonia-lyase [9] and tryptophan decarboxylase [12] make it likely that not only the availability of substrate but also the activities of key enzymes are involved in this regulation.

For higher plants also light is a potent factor in secondary metabolism both in intact plants and tissue cultures. In callus tissue of *Catharanthus roseus* the light-dependent formation of serpentine [3] and anthocyanins [1] has been described. As it is shown by our results with suspension cultures which have a more defined medium environment, the light effect was largely dependent on the medium composition, i.e. the optimal effect of light to stimulate the formation of anthocyanins and serpentine required low concentrations of 2,4-D, phosphate and mineral nitrogen. Thus, the influence of light under our conditions can be separated as a modifying effect on the nature of the main alkaloids induced by the medium effect which does not require light, and as a quantitative stimulation and qualitative alteration (formation of anthocyanins) of phenolic metabolism. From the time-course of alkaloid accumulation (Fig. 1), it can be seen that the first alkaloid produced is ajmalicine as also occurs in the dark [4]. Later on a decreasing level of ajmalicine and increasing amounts of its oxidation product serpentine point to, but do not prove, a conversion of one compound into the other

under the influence of light. On the other hand, the inhibition of secondary product formation by phosphate was less pronounced in illuminated cultures also indicating a general activation of secondary metabolism by light. In cell cultures of other plants the biosynthesis of anthocyanins also was stimulated by light [6, 13, 14] and sucrose [14] whereas inhibitory [14] or no [13] effects were reported for 2,4-D. As shown here, apart from phytohormones, very basic nutrients such as phosphate and nitrogen-containing mineral salts can interfere with the light-induced formation of anthocyanins. These minerals affected the accumulation of anthocyanins (90% inhibition) more than the content of total phenolics (*ca* 50% inhibition). On the other hand, high sucrose concentrations preferentially enhanced the levels of anthocyanins and serpentine having less effect on total phenolics and alkaloids. Thus, the later, light-dependent biosynthetic steps seem to be more susceptible to the medium composition.

It has been reported that by selection of variant strains of *Catharanthus* cell cultures either serpentine- or ajmalicine-producing strains can be obtained [8]. As shown here, the formation of these two products can be also influenced by environmental factors such as medium composition and light. The specific contents of alkaloids produced by our cultures are similar to those found in roots of intact plants [8]. It must be noted that these high levels of alkaloids are formed and accumulated by a minority of the cells present in the culture. It remains a promising goal to elucidate the reasons for this striking heterogeneity. It can be hoped that suitable selection procedures [15] and optimization of culture conditions will lead to cell cultures having higher contents and a larger spectrum of alkaloids.

EXPERIMENTAL

Cell material. Cell suspension cultures of *Catharanthus roseus* (L.) G. Don, strain CP3, were maintained and subcultured as described in ref. [4]. Cell suspensions were illuminated continuously after cell transfer for medium induction with fluorescent lamps (Philips, 40 W/18) at an intensity of *ca* 2800 lx.

Analytical procedures. Extraction and quantitative determination of the alkaloids ajmalicine and serpentine and of phenolic compounds (Folin test) were done according to ref. [4]. For anthocyanin determination 0.5 g (fr. wt) cells were extracted with 10 ml MeOH–HCl (0.01%) at 4° overnight. After filtration the absorbance at 530 nm was measured. Molar anthocyanin contents were calcd using an ϵ of 33000 [6].

Serpentine was identified and distinguished from alstonine by (a) TLC on Si gel in solvents A (C_6H_6–Me_2CO–Et_2NH, 7:2:1) and B (EtOH–EtOAc–Et_2NH–DMF, 6:3:0.5:2); the product from the cells co-chromatographed with serpentine, whereas alstonine had a different R_f; (b) after purification by prep. TLC in solvent A a NMR spectrum was recorded and found to be identical with serpentine and (c) after reduction of the purified compound with $NaBH_4$ only ajmalicine could be identified by TLC in solvents A and C ($CHCl_3$–MeOH, 95:5) and D (xylol–MeCOEt–Et_2NH, 2:1 + 2%). If alstonine was present the reduction product tetrahydroalstonine would have been found.

For the identification of the anthocyanidins, extracts from illuminated cell cultures and from petals of *Catharanthus roseus* were prepared and hydrolysed as described in ref. [16]. For HPLC an LDC Model II G constametric pump with a Rheodyne Model 7120 syringe-loading injector was used. Detection was effected by means of an LDC Model 1204 spectromonitor at 530 nm. Chromatography was carried out on Li-Chrosorb RP-8/7 μm (250 × 4 mm; E. Merck, Darmstadt) with the gradient system from ref. [17]; however, the final concn of solvent B was 70%. PC separation of the anthocyanidins was performed on pre-washed Whatman 3 MM with solvent E (HCOOH–HCl–H_2O, 5:2:3) [18]. The separated anthocyanidins were cut out and eluted with MeOH–HCl (0.01%). Further characterization by cellulose-TLC was done in solvents F (HOAc–HCl–H_2O, 30:3:10) and G (*n*-BuOH–HOAc–H_2O, 4:1:5; upper layer) [18].

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