Influence of Medium Composition on the Formation of Secondary Compounds in Cell Suspension Cultures of *Catharanthus roseus* (L.) G. Don

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Cell suspension cultures of *Catharanthus roseus* have been subjected to various media conditions in order to stimulate the formation of indole alkaloids. High ajmalicine contents (up to 0.5 mg/g cell fresh weight) were achieved by transferring 2-week-old cell suspensions to a 10-fold volume of a 8% sucrose solution. The alkaloid accumulation started two days after the transfer and reached a plateau after ten days. Furthermore an enhanced level of phenolic compounds was found, whereas growth of the culture was low. The accumulation of both, alkaloids and polyphenols was stimulated by high concentrations of sucrose and low concentrations of nitrogen containing salts and phosphate. When these minerals were added to the sucrose solution in concentrations commonly used for cell culture media, the accumulation of alkaloids and phenolic compounds was largely suppressed.

Introduction

Over 100 different indole alkaloids have been isolated from the periwinkle plant (*Catharanthus roseus* (L.) G. Don), among them the quantitatively important ajmalicine-type alkaloids ajmalicine and serpentine in yields up to 0.9% on dry weight basis [1]. In contrast, only small amounts of these alkaloids have been found at first in cell cultures of this plant [2]. In the recent years considerable success has been achieved in increasing the alkaloid content in *Catharanthus roseus* cell suspension cultures by selection of high producing strains [1] and variation of some constituents of the nutritional media *e. g.* phytohormones, carbon sources and addition of precursors [1, 3–5]. Few attention, however, has been given so far to the influence of the inorganic salts that are essential constituents of all media for plant cell culture, although it is known from the work on microorganisms [6], that these compounds can have pronounced effects on the production of secondary metabolites. From this point of view we wish to report further results to the improvement of culture conditions favouring the accumulation of secondary compounds in cell suspension cultures of *Catharanthus roseus.*

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthalene acetic acid; BAP, benzylaminopurine.

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Materials and Methods

Suspension cultures of Catharanthus roseus (L.) G. Don

Catharanthus roseus cell suspension cultures were maintained and subcultured every 2 weeks in a medium described by Murashige and Skoog [7] (MS-medium) with the addition of 3% sucrose and 2×10^{-6} M 2,4-dichlorophenoxyacetic acid with 2 g cells (fresh weight) as inoculum. The cells were grown in 200-ml-Erlenmeyer flasks containing 70 ml of culture medium at 27 °C in the dark. The flasks were shaken at 110 strokes/min on a gyratory shaker. For experiments 7 ml of a 2-week-old culture were transferred to 70 ml of the tested medium. The pH of all test media was adjusted to pH 6. Normal incubation time in this medium was another 2 weeks. Cells were harvested by vacuum filtration through a porous glass filter and immediately extracted for alkaloids and phenolic compounds. All experiments were performed using strain CP3 which was derived from stem pieces of germinating seedlings.

Analytical procedures

Ajmalicine and serpentine

Freshly harvested cell material (0.5 g fresh weight) was extracted with 10 ml 70% ethanol (v/v) for 2 h at 60 °C. For the determination of the alkaloids in the medium, 3 ml of filtrated medium were mixed with 7 ml ethanol. The alcoholic solutions were

diluted with water, alkalinized with Na_2CO_3 and extracted with methylene chloride. An aliquot of the organic phase was used for the direct fluorometric determination of serpentine (excitation at 309 nm, emission at 444 nm) in a double-monochromator spectrofluorometer JY 3 (Jobin Yvon, Longjumeau/France). Another aliquot of the organic phase was chromatographed on silica gel plate with chloroform-methanol 95:5 (v/v). Ajmalicine spots were cut out and eluted with 1.5 ml 1% H_2SO_4 in methanol-water 1:1 (v/v). 1 ml of this solution was mixed with 0.1 ml 0.5% ceric ammonium sulfate in 2% H_2SO_4, diluted with 2 ml 1% H_2SO_4 and after 5 min 0.05 ml of 1% H_2O_2 were added. Ajmalicine was thus oxidized to serpentine and subsequently determined as described above. Standards of authentic ajmalicine and serpentine were treated similarly and used as reference solutions for the fluorometric determination. All quantitative alkaloid values are given as weight of free alkaloid base relating to cell fresh weight.

Polyphenols and growth

Determination of the polyphenol content of the cells was done by the Folin-method as previously described [8]. Growth is expressed as percentage of cell fresh weight increase relating to initial cell mass.

Results

Accumulation of alkaloids

When cells of *Catharanthus roseus* were grown in MS-medium [7] supplemented with 2×10^{-6} M 2,4-D accumulation of the indole alkaloids ajmalicine and serpentine was low (Table I). By omitting 2,4-D from the medium enhanced levels of these alkaloids, mainly ajmalicine were observed. A similar increase in alkaloid accumulation was found by transferring the cells to the alkaloid production medium described by Zenk and coworkers [1]. Highest alkaloid levels, however, were achieved when cells were first grown in MS-medium containing 2×10^{-6} M 2,4-D and subsequently transferred to a 10-fold volume of a 8% sucrose solution in water. It must be stressed that in all experiments alkaloid content is expressed in relation to cell fresh weight (see discussion). The addition of the phytohormones of the alkaloid production medium [1] and/or the feeding of the biosynthetic precursor L-tryptophan in 2.5×10^{-3} M concentration to the sucrose solution caused a decrease of the alkaloid content compared to the induction by sucrose alone. In every experiment ajmalicine was the predominant alkaloid produced whereas serpentine reached only up to about 10% of the ajmalicine value. Preliminary results indicate, that a large number of other *Catharanthus* alkaloids was formed together with ajmalicine and serpentine. Sucrose could be replaced by other mono- or disaccharides with various efficiency for the alkaloid formation (Fig. 1). Equimolar solutions of glucose or maltose were found to be equally effective, whereas cells transferred to solutions of fructose, galactose or lactose accumulated about half the amount of ajmalicine and serpentine compared to sucrose.

The induction effect of sucrose solutions on alkaloid accumulation was greatly dependent on the sucrose concentration (Fig. 2). Solutions of 8% (w/v) sucrose were found to be optimal, whereas at zero concentration (dilution into water) no significant alkaloid formation could be observed (Table I). A time course of the alkaloid accumulation in 8% sucrose is shown in Fig. 3. After the cell transfer there was a lag-phase of 2 days, after which the ajmalicine accumulation started. From day 2 until

Table I. Influence of various media conditions on the growth and relative alkaloid content (sum of serpentine and ajmalicine) in cell suspension cultures of *Catharanthus roseus*. Cell suspensions grown in MS-medium containing 2×10^{-6} M 2,4-D were diluted into 10-fold volumes of the various media and harvested after two weeks.

Medium	Relative alkaloid content [%]	Growth [%]
MS-medium + 2×10^{-6} M 2,4-D	6.7	1260
MS-medium [7]	36.6	719
"Production medium" [1]	45.5	436
H_2O	< 2	39
8% Sucrose	100.0	22
8% Sucrose + 2.5×10^{-3} M L-tryptophan	3.1	< 0
8% Sucrose + 10^{-6} M IAA + 5×10^{-6} M BAP	75.0	< 0
8% Sucrose + 2.5×10^{-3} M L-trp + 10^{-6} M IAA + 5×10^{-6} M BAP	9.8	< 0

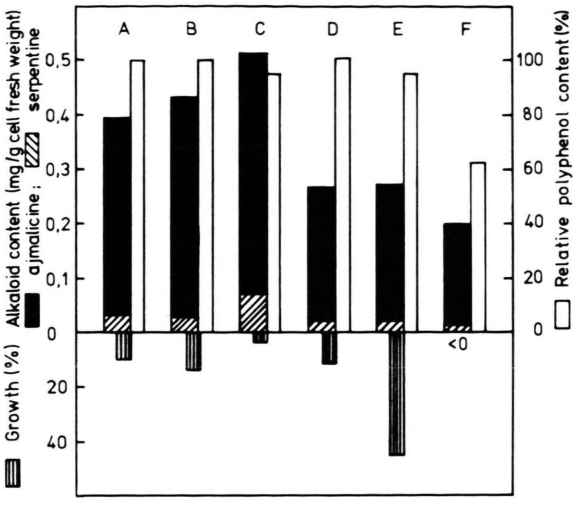

Fig. 1. Influence of various sugars on cell growth and formation of secondary compounds. Cells grown in MS-medium containing 2×10^{-6} M 2,4-D were diluted in a 10-fold volume of the indicated sugar solution (0.23 M in water). Incubation time was 2 weeks. A, sucrose, B, glucose; C, maltose; D, fructose; E, galactose; F, lactose.

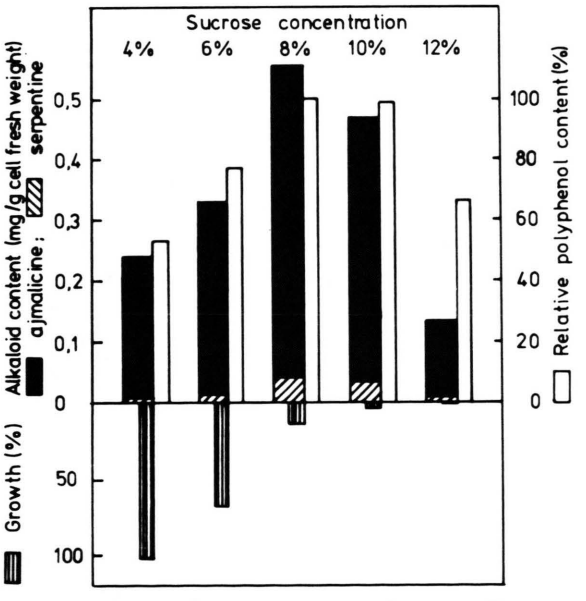

Fig. 2. Influence of sucrose concentrations on cell growth and formation of secondary compounds. Cells were diluted in a 10-fold volume of an aqueous sucrose solution and harvested after 2 weeks.

day 10 the ajmalicine concentration rose continuously. After 10 days a maximum level was reached. After day 2 a very low but continuously increasing serpentine level was observed.

In order to investigate the influence of inorganic salts (macronutrients) of the standard culture media on the alkaloid formation some of these constituents were added to the sucrose induction medium. The results are shown in Fig. 4. Nitrogen containing mineral salts (composition identical to MS-medium) or phosphate were able to suppress almost completely the accumulation of the alkaloids. These compounds were added in the same concentrations that are used in the MS-medium. Two other mineral salts of the MS-medium, $CaCl_2$ and $MgSO_4$ did not interfere with the alkaloid accumulation. The addition of all other mineral salts (micronutrients) and vitamins of the MS-medium did not suppress alkaloid accumulation.

In most of the experiments described here, the alkaloid content was measured separately in the medium and in the cells. In no case a significant (*i. e.* greater than 5%) amount of ajmalicine and/or serpentine was found in the medium, indicating that under the experimental conditions used, these alkaloids were accumulated within the cells and not excreted to the medium. This is also an indication that no significant lysis of cells had occured.

Accumulation of phenolic compounds

Phenolic compounds are secondary plant products, which originate from different biochemical pathways than the indole alkaloids. Therefore the influence of media conditions favouring the accumu-

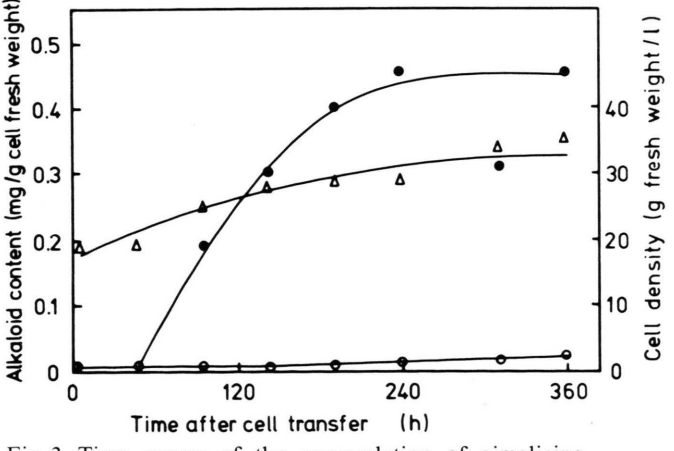

Fig. 3. Time course of the accumulation of ajmalicine (●–●) and serpentine (○–○) after dilution of *Catharanthus roseus* cells into a 8% aqueous sucrose solution. Cell density (△–△) is indicated as cell fresh weight/culture volume.

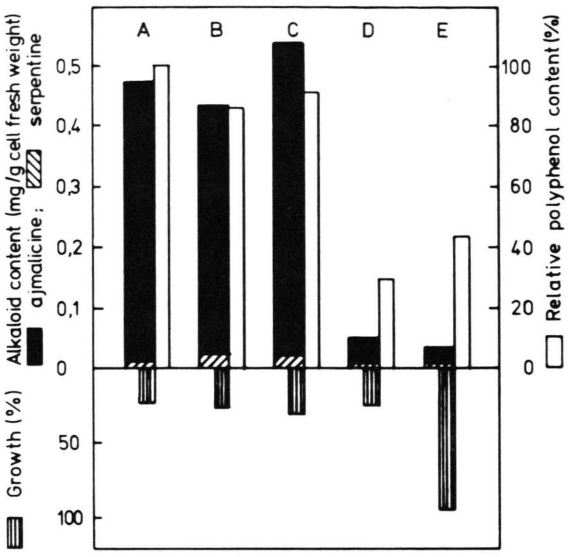

Fig. 4. Influence of various mineral salts on the production of secondary compounds induced by medium dilution and sucrose. The following salts have been added: A, no addition (control); B, $CaCl_2$ (3×10^{-3} M); C, $MgSO_4$ (1.5×10^{-3} M); D, KNO_3 (18.9×10^{-3} M) together with $(NH_4)NO_3$ (20×10^{-3} M); E, KH_2PO_4 (1.2×10^{-3} M).

lation of alkaloids was also investigated with regard to the accumulation of polyphenols (Figs. 1, 2 and 4). In all experiments there was a parallel increase in the content of alkaloids and phenolic compounds. This was most obvious for the dependence on the sucrose concentration (Fig. 2) and the inhibition of induction by nitrogen containing mineral salts or phosphate (Fig. 4).

Growth

In most of the media which were used to stimulate the formation of secondary products the increase of cell fresh weight was very low (Table I). This reduced growth can be seen quite clearly from the time course of cell mass and secondary product accumulation of a culture in 8% sucrose (Fig. 3). This culture contained only the minerals, phytohormones and vitamins, which were transferred from the parent culture with the inoculum volume. Thus the low concentrations of these compounds probably limit growth. The addition of phosphate to the sucrose medium did not only suppress secondary product formation but stimulated the growth of the culture significantly (Fig. 4). Another factor influencing the increase of cell mass was the sucrose concentration (Fig. 2). Under otherwise identical conditions of nutrient deficiency high sucrose concentrations inhibited culture growth.

Discussion

Since the early work on nutritional requirements of plant cell cultures [9] cell culture media have been designed mainly to support growth. From the work on microorganisms, however, it is known that growth and production of secondary compounds often exclude each other in a way, that maximal production does not occur until growth of the culture decreases before reaching the stationary phase [6]. A similar behaviour has been observed at least for some plant cell cultures producing secondary compounds *e. g. Haplopappus gracilis* [10], *Rosa* cells [11], *Acer pseudoplatanus* [12] and *Morinda citrifolia* [13]. In soybean cell suspension cultures enzymes of secondary metabolism show maximal activity at the beginning of the stationary phase [14]. In fermenter cultures of *Catharanthus roseus* accumulation of serpentine does not start until the stationary phase of the culture is reached [1, 5]. In flask cultures, however, growth and alkaloid accumulation parallel each other. In a recent study of the influence of low temperatures on suspension cultures of *Catharanthus roseus* [15] growth and alkaloid formation were found to be inversely correlated. From these results it seemed worthwhile to investigate media conditions, which were far away from being optimal for growth and thus to get more information on the nutritional requirements for the production of secondary compounds. As our main interest was directed to metabolic changes of the cells with regard to the formation of secondary products, alkaloid content was expressed as specific alkaloid content *i. e.* in relation to cell fresh weight and not to culture volume, in order to eliminate the influence of growth to a large extend.

The results presented here give further indication that the formation of secondary compounds can be stimulated by the use of rather unusual nutrient conditions, which limit growth considerably. Indole alkaloids and phenolic compounds, which have been produced under these conditions originate at least partially from amino acids which in turn are needed by rapidly growing organisms for the synthesis of proteins. From this point it seems reasonable to assume a competition of two

biochemical pathways for one common precursor [12]. Conditions, which suppress one pathway may therefore be favourable for the other. As it is discussed by Phillips and Henshaw [12], it is difficult to verify this precursor competition hypothesis by the examples cited in the literature [10, 11–13]. It cannot be ruled out that the cell cultures under study contained various cell populations in different stages of the cell cycle [12]. This inhomogeneity could be favoured by the slowly changing environmental conditions in a batch culture. Therefore formation of secondary products during culture growth could be due to cells, which had already entered the stationary phase. In our experiments the rapid change of the environmental conditions presumably synchronized this event, leading to a sudden onset and a high rate of secondary product formation. These results indicate an antagonistic regulation of growth and secondary metabolism, as it is discussed in the precursor competition hypothesis.

Several factors were found to favour secondary product formation: Low concentrations of 2,4-D and of some mineral salts as well as high concentrations of sucrose. The beneficial effect of sucrose on the formation of secondary compounds in cell cultures has been reported by several authors before [12, 13, 16, 17]. When *Catharanthus* cells were grown in standard liquid media, 5% sucrose were found to stimulate the accumulation of indole alkaloids [1, 5]. At present, however, we cannot yet decide whether the sucrose effect is based on a direct metabolic influence or whether it is a consequence of osmotic effects.

The inhibition of secondary product formation by auxins like 2,4-D is a very often observed phenomenon and has been explained by its action on protein turnover [12]. The influence of a broad spectrum of phytohormones on the formation of alkaloids in *Catharanthus* cell cultures was studied and as a result a special "production medium" was formulated [1]. In this medium an increased alkaloid formation but a reduced growth was observed. Our cell line responded similarly to the production medium. However, the alkaloid content did not reach the level achieved by dilution of the cells with 8% sucrose (Table I).

In contrast very few results on the influence of inorganic salts on secondary metabolism in plant cell cultures have been published so far, although the effect of these compounds on the production of secondary metabolites by microorganisms has been investigated very thoroughly (reviewed in ref. [6]).

The influence of low mineral salt concentrations on the alkaloid synthesis in *Tylophora indica* [18], and of phosphate concentrations on ubiquinone formation in tobacco cell suspension cultures [19] has been studied previously, but no effects have been found on the production of secondary compounds. These results may be due to the rather narrow concentration ranges used in these experiments. Low medium nitrogen concentrations were found favouring the xylem cell differentation in jerusalem artichoke cells [20], an example for the effect of mineral salt concentration on cytodifferentiation and probably secondary metabolism.

For the formation of secondary compounds by *Catharanthus* cells phosphate and nitrogen containing mineral salts seem to play an important role, since addition of these compounds to the induction medium, containing only sucrose, largely suppressed the formation of alkaloids and polyphenols. Other constituents of the MS-medium did not affect secondary product formation. On the other hand the addition of phosphate stimulated the growth of the culture significantly. A very similar effect of phosphate was observed for the production of the antibiotic tylosin by *streptomyces* [21]. This is another indication, that culture growth and production of secondary compounds may be regulated antagonisticly. If this observation is also valid for other cell cultures, and we have experimental evidence that this holds true (Knobloch and Berlin, unpublished results), these results may give a general explanation for the low levels of secondary products in rapidly growing plant cultures. The mechanism, however, of switching from growth and primary metabolism to the production of secondary compounds remains to be elucidated and for this purpose a definite "induction system" like the one described can be valuable tool.

From the results presented here the following conclusions can be drawn: Aside from the selection of high producing strains the idea of different media for growth and production has to be expanded not only with regard to the phytohormone composition but also to very basic components like mineral salts and carbon sources. This may include the exchange of a liquid culture medium during the growth cycle of a cell culture and further work is in progress to solve the biotechnological problems of this process.

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