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IMPROVED PRODUCTION OF RAUCAFFRICINE BY CULTIVATED *RAUWOLFIA* CELLS

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Key Word Index—*Rauwolfia serpentina*; Apocynaceae; cell suspension culture; indole alkaloids; raucaffricine production; vomilenine.

Abstract—The production of the glucoalkaloid raucaffricine has been optimized in cell suspension cultures of *Rauwolfia serpentina*. In an alkaloid production medium modified by adding 100 g sucrose and 2.5 g $MgSO_4 \cdot 7H_2O$/l of medium, the culture produced after 18 days up to 1.6 g raucaffricine when 1 l medium was inoculated with 200 g of cells grown for 10 days in Linsmaier–Skoog medium. This yield exceeds that known from intact plants (*Rauwolfia caffra*) by a factor of 12 and is the fifth highest value for a secondary plant metabolite produced by cultivated plant cells.

INTRODUCTION

Plant cell suspension cultures of *Rauwolfia serpentina* (L.) Benth. have proved to be an excellent source of the enzymes involved in the biosynthesis of the *Rauwolfia* alkaloids of the ajmalan and sarpagan class [1]. Moreover, a phytochemical characterization of cell cultures based on their capability for indole alkaloid formation demonstrated that this culture is the most efficient of the presently known systems. So far, we have isolated and identified 26 different indole alkaloids from cultivated Rauwolfia cells [2]. Alkaloid production under optimum conditions ranges from μg to g/l of medium.

Recently, we have focused our attention on the optimization of the formation of the major alkaloid of *R. serpentina* cultures, i.e. the glucoalkaloid raucaffricine (vomilenine-β-D-glucopyranoside). Its aglycone vomilenine, which can be easily generated by raucaffricine-β-D-glucosidase, occupies a branch-point position in the ajmaline pathway. Because we are interested in using multifunctional biosynthetic intermediates such as vomilenine as synthons for biomimetic syntheses, high production rates of the required alkaloid or its glycoside are desirable. In this paper we describe the optimization of raucaffricine synthesis in suspended *Rauwolfia* cells.

RESULTS AND DISCUSSION

Our previous observation [3] that *R. serpentina* cell suspension cultures produce the glucoalkaloid raucaffricine as the major alkaloid was surprising because this alkaloid has been isolated in the past not from this species but exclusively from *R. caffra* plants [4, 5 and W. E. Court, personal communication]. The detection of the enzyme raucaffricine-β-D-glucosidase [6] indicated, however, that the occurrence of the glucoalkaloid in *R. caffra* plants might not be an exception because it is this enzyme and the treatment of the plant material before alkaloid isolation which determine the raucaffricine content. Since usually only dried plant material is available for alkaloid extraction, the degradation of raucaffricine by the glucosidase can be expected to take place to a large extent, this is a general problem in the isolation of glycosides. When we analysed air-dried cell material of the *Rauwolfia* culture for raucaffricine, its content was less than 30% compared with fresh cells, an observation which was also made with *R. caffra* leaves (W. E. Court, personal communication). Therefore we always used cell material which had been stored for only a few days at −25° and was then freeze-dried. Only under these conditions was the raucaffricine content of the cells unaffected.

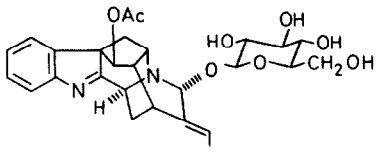
In order to select the most productive cell material and to develop an optimization procedure for raucaffricine production, we analysed well-established cell suspensions of *Rauwolfia* species. As shown in Table 1, the four species tested produced the glucoalkaloid in a standard growth medium (LS-medium). This result shows that raucaffricine is not restricted exclusively to intact plants of *R. caffra*. The highest raucaffricine value (nearly 0.4 g/l medium) observed in *R. serpentina* cell suspensions exceeded all others by a factor of more than 10, although the cell mass was only 20 g dry wt/l of medium. All the following investigations were therefore carried out exclusively with this culture.

When *R. serpentina* cells were grown for 18 days in standard nutrition media such as 4X, DAX, Ringe or AP-medium the cell dry wt and alkaloid formation could be increased in the alkaloid production (AP) medium up to 27 g and 0.6 g/l respectively.

The formation of natural products by cell cultures is frequently dependent on the supply of carbohydrates. Although large amounts of sucrose for instance were used to enhance the production of rosmarinic acid [7], the influence on anthraquinone formation in 19 cell cultures of *Rubiaceae* species was unpredictable [8]. We studied the effect of galactose, glucose and sucrose on alkaloid formation in *Rauwolfia* cells by using AP-medium containing increased amounts of carbohydrates (Fig. 1). In the case of galactose, optimum raucaffricine synthesis was reached at 75 g/l. However, under these conditions only

Table 1. Formation of raucaffricine in suspension cultures of different *Rauwolfia* species after 18 days growth in Linsmeyer–Skoog medium.

Cell culture	Cell dry wt (g/l)	Raucaffricine (mg/l)	Raucaffricine (% dry wt)
R. verticillata Chevalier	13	22	0.17
R. mannii Stapf	17	13	0.08
R. serpentina Benth.	20	390	1.95
R. caffra Sond.	30	53	0.18



Raucaffricine

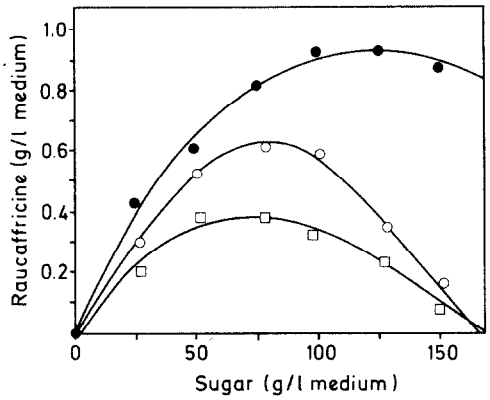


Fig. 1. Dependence of raucaffricine formation on the carbohydrate source. ●——●, Sucrose; ○ ····○, glucose; □——□, galactose.

0.35 g of the glucoalkaloid was synthesized per l. In contrast, 0.6 g raucaffricine was obtained at the same glucose concentration. When glucose was replaced by sucrose, alkaloid synthesis was increased up to 0.8 g/l and reached the maximum between 100 and 125 g sucrose with *ca* 0.9 g/l raucaffricine. During these investigations, it was observed that at a high concentration of glucose (150 g/l) the formation of strictosidine strongly increased up to 0.5 g/l medium at the expense of raucaffricine production. Only 0.15 g/l of the last named compound were formed. Although the activity of strictosidine-glucosidase can be inhibited in crude enzyme preparations by high concentrations of glucose (50% inhibition at 4 M glucose), it is not yet clear whether the *in vivo* accumulation of strictosidine is exclusively due to this inhibitory effect. It is, however, an interesting and very rare example of the modulation of a pathway by a medium component. Based on the above medium composition (AP-medium with 100 g sucrose (API)) we tried to optimize alkaloid formation further by variation of the medium constituents.

Another way of increasing the production of a secondary metabolite is by changing the concentration of different mineral salts. A well known example is the formation of shikonin derivatives in cell suspensions of *Lithospermum erythrorhizon* which strongly depends on the concentration of Cu^{2+} ions [9]. Ten mineral salts, each of which was tested at a concentration of 10 mM had a marked effect on cell growth and raucaffricine production (Table 2). In the presence of $CoCl_2$, $CuSO_4$ or $ZnCl_2$ less than 30% of the optimum cell mass could be obtained with negligible alkaloid formation ($< 1.4 \times 10^{-3}$ g/l); cells treated with $CaCl_2$ and $MgSO_4$ showed excellent raucaffricine formation of > 1 g/l (57 g dry cells/l).

Maximum alkaloid content (1.2 g/l) was observed after a growth period of 18 days (Fig. 2). These conditions (AP-medium, 100 g sucrose, 10 mM $MgSO_4$ (APII)) were routinely used for raucaffricine production because the influence of other nutritional components like, L-tryptophan or *myo*-inositol was relatively small. This optimum production of raucaffricine has now be monitored over a period of four years.

A final optimization was achieved by variation of the amount of inoculum. Sometimes maximum values of up to 1.6 g/l raucaffricine were found when 200 g cells grown for 10 days in LS-medium were transferred to 1 l of the optimized alkaloid production medium. These values were however hard to reproduce.

If one compares the raucaffricine production rates observed with the different media (Fig. 3), it is quite clear that alkaloid production is proportional to cell growth. The productivity with the currently described modified AP-medium is sufficient for the isolation of raucaffricine in gram amounts by rotation locular counter current chromatography (RLCC). In the past, we have isolated *ca* 100 g of the glucoalkaloid for biomimetic syntheses of ajmalan and sarpagan alkaloids and therefore did not try to increase the cellular alkaloid content further by other methods, such as strain selection. If, however, one takes into consideration the highest raucaffricine values so far measured, the cell suspension culture method on a dry wt

Table 2. Influence of mineral salts on cell growth of *R. serpentina* cell suspensions and raucaffricine formation (cells were grown in 75 ml API-medium for 23 days).

Mineral salts (10 mM)	Cell dry wt (g/l)	Raucaffricine (g/l)
$CoCl_2$	7.4 ± 0.7	0
$CuSO_4$	10.9 ± 0.5	1.4×10^{-3}
$ZnCl_2$	12.4 ± 0.1	0.5×10^{-3}
K_2HPO_4	48.7 ± 5.2	0.53
NaCl	54.1 ± 0.2	0.78
$MgCl_2$	54.6 ± 0.7	0.78
$MnSO_4$	54.8 ± 0.8	0.81
$CaCl_2$	56.1 ± 0.8	1.07
Na_2SO_4	56.9 ± 3.2	0.85
$MgSO_4$	58.5 ± 1.4	1.04

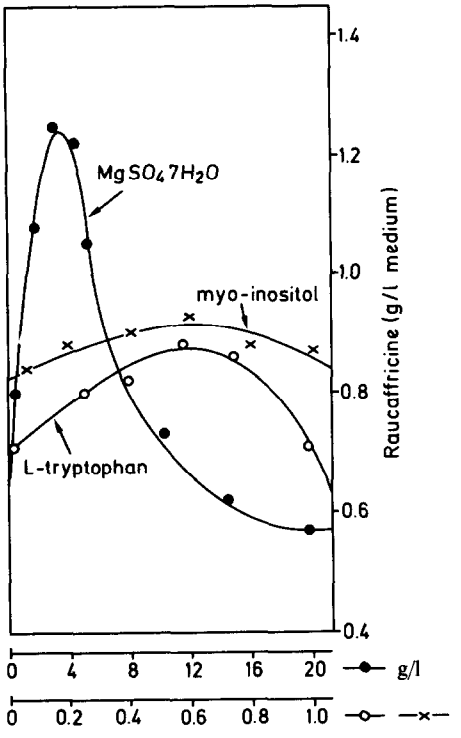


Fig. 2. Influence of L-tryptophan, *myo*-inositol and $MgSO_4$ on the production of raucaffricine.

basis already exceeds by twelve-fold the best natural raucaffricine source (*R. caffra* plants).

Our final aim of employing the branchpoint intermediate vomilenine (raucaffricine aglycone) as a synthon for biomimetic syntheses has been reached by the production of raucaffricine combined with the efficient enzymatic liberation of vomilenine. This reaction is catalysed by raucaffricine-β-D-glucosidase. Vomilenine can be obtained in 85% yield if the same crude protein mixture (0.15 g) from 0.4 kg fresh *Rauwolfia* cells is incubated with

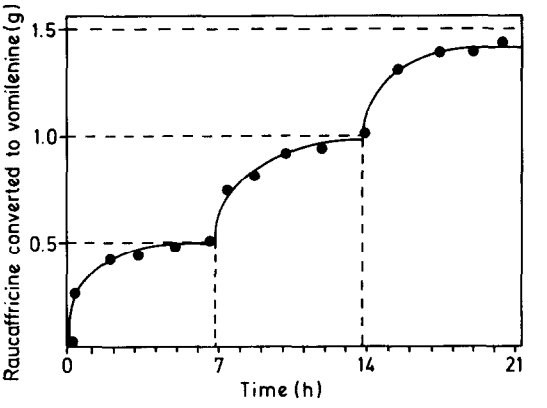


Fig. 4. Enzymatic conversion of raucaffricine into its aglycone vomilenine catalysed by a crude raucaffricine-β-D-glucosidase under continuous extraction with ethyl acetate.

each 0.5 g raucaffricine for 7 hr (3 × 0.5 g of the glucoalkaloid were converted) under continuous extraction of the total mixture with ethyl acetate (Fig. 4). Although the conversion rate decreased after the third addition of the glucoalkaloid due to denaturation of the glucosidase by the organic solvent, the procedure is now used in our laboratory for routine vomilenine preparation.

EXPERIMENTAL

Cell cultures. Cell suspensions of *Rauwolfia caffra* Sond., *R. mannii* Stapf., *R. serpentina* Benth. and *R. verticillata* Chevalier were maintained with shaking (100 rpm) in 1 l Erlenmeyer-flasks each of which contained 250 ml Linsmaier–Skoog medium [10]. Subculturing was performed at weekly intervals. For storage of cell material cells were frozen with liquid N_2 and stored at −25° for enzyme isolation. For extraction of alkaloids the cells were freeze-dried. In addition the following nutrition media were used: 4X [11] modified as described in [12], DAX [13], Ringe-medium [14], AP-medium [13].

HPLC. The raucaffricine content of the *Rauwolfia* cell cultures was determined by HPLC. A Spectra Physics instrument (SP

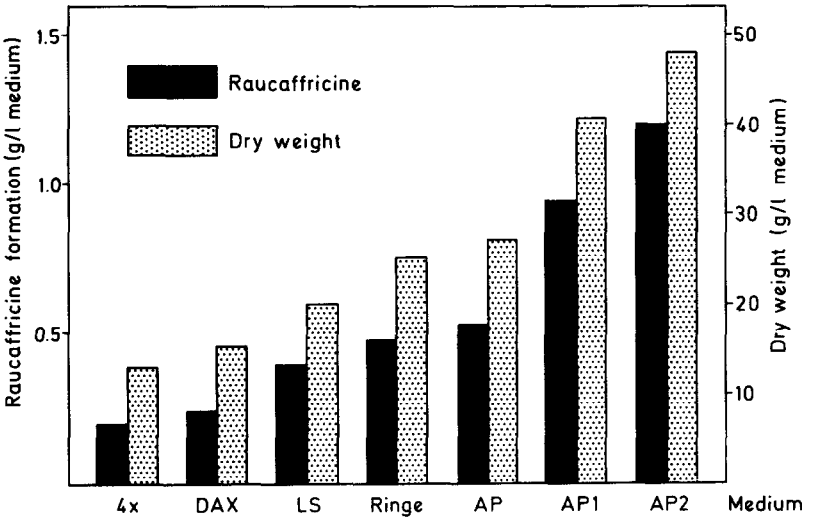


Fig. 3. Optimization of raucaffricine production and cell growth by using different standard nutrition media.

8440 UV/VIS detector, SP 4100 computing integrator, SP8700 solvent delivery system, and SP8780XR autosampler) was used in combination with an RP-18 column (0.4 × 25 cm) Hibar LiChroCart, 7 μm; method (a): MeCN–10 mM $(NH_4)_2CO_3$ (1:4→3:2 within 30 min); method (b): a RP-select B column (0.4 × 25 cm) Hibar LiChrosorb with (1) MeCN–K-Pi buffer (6.66 g KH_2PO_4, 2.8 ml H_3PO_4 (85%) made up to 1 l) (1:9) (2) same constituents but ratio 3:7. Solvent mixture (1)/solvent mixture (2) (3:2→1:3 within 20 min). Flow rate was 1 ml/min and UV detection at 280 nm (method a) or at 258 nm (method b).

Alkaloid isolation was performed by standard isolation procedures, preferentially by rotation locular counter current chromatography (RLCC) if preparative amounts were needed. An RLCC instrument Zinsser Analytik GmbH Frankfurt/Main was employed. The freeze-dried cell material (0.5 kg) was extracted (× 2) with MeOH for 2 days. The organic phase was concd to 0.1 l, diluted with 0.9 l H_2O and after centrifugation passed through a XAD-2 column (5 cm × 41 cm) at a flow rate of 200 ml/hr. After washing the column with 1.5 l H_2O, the adsorbed alkaloids were eluted with 1.5 l MeOH. The organic solvent was evapd to dryness. The concd alkaloid mixture was separated by RLCC after dissolving in the mobile phase under the following conditions: ascending mode, $CHCl_3$–MeOH–H_2O (43:37:20) (organic phase = stationary phase; aqueous phase = mobile phase); the rotor slope was 35° and rotation was performed at 80 rpm. The flow rate was 1.0 ml/min and fractions of 10 ml were collected. The fractions were analysed by TLC [solvent system (1): petrol–Me_2CO–Et_2N (7:2:1); solvent system (2): $CHCl_3$–MeOH–NH_3 (4:1:0.01)].

Formation of vomilenine. A crude enzyme extract (0.15 g protein) of an *R. serpentina* cell culture grown for 18 days in AP-medium was incubated in the presence of 300 ml K-Pi buffer (0.1 M, pH 7.0, 10 mM 2-mercaptoethanol) and 0.5 g raucaffricine under continuous extraction with EtOAc. After 7 and 14 hr, 0.5 g amounts of raucaffricine were added and the procedure repeated. After evapn of the organic solvent, 0.87 g vomilenine (85%) were obtained.

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